

#### **55P4H4: GENE EXPRESSED IN VARIOUS CANCERS**

This application claims the benefit of United States provisional application number 60/211,454, filed June 13, 2000, the entire contents of which are incorporated herein by reference.

#### **FIELD OF THE INVENTION**

5 The invention described herein relates to a novel gene and its encoded protein, termed 55P4H4, and to diagnostic and therapeutic methods and compositions useful in the management of various cancers that express 55P4H4.

#### **BACKGROUND OF THE INVENTION**

10 Cancer is the second leading cause of human death next to coronary disease. Worldwide, millions of people die from cancer every year. In the United States alone, cancer causes the death of well over a half-million people annually, with some 1.4 million new cases diagnosed per year. While deaths from heart disease have been declining significantly, those resulting from cancer generally are on the rise. In the early part of the next century, cancer is predicted to become the leading cause of death.

15 Worldwide, several cancers stand out as the leading killers. In particular, carcinomas of the lung, prostate, breast, colon, pancreas, and ovary represent the primary causes of cancer death. These and virtually all other carcinomas share a common lethal feature. With very few exceptions, metastatic disease from a carcinoma is fatal. Moreover, even for those cancer patients who initially survive their primary cancers, common experience has shown that their lives are dramatically altered. Many cancer patients experience strong anxieties driven by the awareness of the potential for recurrence or treatment failure. Many cancer patients experience physical debilitations following treatment. Furthermore, 20 many cancer patients experience a recurrence.

Worldwide, prostate cancer is the fourth most prevalent cancer in men. In North America and Northern Europe, it is by far the most common cancer in males and is the second leading cause of cancer death in men. In the United States alone, well over 40,000 men die annually of this disease - 25 second only to lung cancer. Despite the magnitude of these Figures, there is still no effective treatment for metastatic prostate cancer. Surgical prostatectomy, radiation therapy, hormone ablation therapy, surgical castration and chemotherapy continue to be the main treatment modalities. Unfortunately, these treatments are ineffective for many and are often associated with undesirable consequences.

30 On the diagnostic front, the lack of a prostate tumor marker that can accurately detect early-stage, localized tumors remains a significant limitation in the diagnosis and management of this disease. Although the serum prostate specific antigen (PSA) assay has been a very useful tool, however its specificity and general utility is widely regarded as lacking in several important respects.

Progress in identifying additional specific markers for prostate cancer has been improved by the generation of prostate cancer xenografts that can recapitulate different stages of the disease in mice. The LAPC (Los Angeles Prostate Cancer) xenografts are prostate cancer xenografts that have survived passage in severe combined immune deficient (SCID) mice and have exhibited the capacity to mimic the transition from androgen dependence to androgen independence (Klein et al., 1997, Nat. Med. 3:402). More recently identified prostate cancer markers include PCTA-1 (Su et al., 1996, Proc. Natl. Acad. Sci. USA 93: 7252), prostate-specific membrane (PSM) antigen (Pinto et al., Clin Cancer Res 1996 Sep 2 (9): 1445-51), STEAP (Hubert, et al., Proc Natl Acad Sci U S A. 1999 Dec 7; 96(25): 14523-8) and prostate stem cell antigen (PSCA) (Reiter et al., 1998, Proc. Natl. Acad. Sci. USA 95: 1735).

While previously identified markers such as PSA, PSM, PCTA and PSCA have facilitated efforts to diagnose and treat prostate cancer, there is need for the identification of additional markers and therapeutic targets for prostate and related cancers in order to further improve diagnosis and therapy.

#### SUMMARY OF THE INVENTION

The present invention relates to a novel gene, designated 55P4H4, that is over-expressed in multiple cancers listed in Table I. Northern blot expression analysis of 55P4H4 gene expression in normal tissues shows a restricted expression pattern in adult tissues. The nucleotide (Figure 2) and amino acid (Figure 2 and Figure 3) sequences of 55P4H4 are provided. The tissue-related profile of 55P4H4 in normal adult tissues, combined with the over-expression observed in prostate and other tumors, shows that 55P4H4 is aberrantly over-expressed in at least some cancers, and thus serves as a useful diagnostic and/or therapeutic target for cancers of the tissues such as those listed in Table I.

The invention provides polynucleotides corresponding or complementary to all or part of the 55P4H4 genes, mRNAs, and/or coding sequences, preferably in isolated form, including polynucleotides encoding 55P4H4-related proteins and fragments of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more than 25 contiguous amino acids; at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 85, 90, 95, 100 or more than 100 contiguous amino acids of a 55P4H4-related protein, as well as the peptides/proteins themselves; DNA, RNA, DNA/RNA hybrids, and related molecules, polynucleotides or oligonucleotides complementary or having at least a 90% homology to the 55P4H4 genes or mRNA sequences or parts thereof, and polynucleotides or oligonucleotides that hybridize to the 55P4H4 genes, mRNAs, or to 55P4H4-encoding polynucleotides. Also provided are means for isolating cDNAs and the genes encoding 55P4H4. Recombinant DNA molecules containing 55P4H4 polynucleotides, cells transformed or transduced with such molecules, and host-vector systems for the expression of 55P4H4 gene products are also provided. The invention further provides antibodies that bind to 55P4H4 proteins and polypeptide fragments thereof, including

polyclonal and monoclonal antibodies, murine and other mammalian antibodies, chimeric antibodies, humanized and fully human antibodies, and antibodies labeled with a detectable marker.

The invention further provides methods for detecting the presence and status of 55P4H4 polynucleotides and proteins in various biological samples, as well as methods for identifying cells that  
5 express 55P4H4. A typical embodiment of this invention provides methods for monitoring 55P4H4 gene products in a tissue or hematology sample having or suspected of having some form of growth dysregulation such as cancer.

The invention further provides various immunogenic or therapeutic compositions and strategies for treating cancers that express 55P4H4 such as prostate cancers, including therapies aimed at inhibiting  
10 the transcription, translation, processing or function of 55P4H4 as well as cancer vaccines.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1. The 55P4H4 SSH-sequence (SEQ ID NO: 3). The SSH experiment was performed with cDNA digested with DPN II. The 55P4H4 sequence contains 300 bp.

15 Figure 2A-C. The cDNA (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequences of 55P4H4. The sequence surrounding a potential start ATG (ACC ATG G) (SEQ ID NO: 4) exhibits a potential Kozak sequence (A at position -3). The putative start methionine and Kozak sequence are indicated in bold. A GC rich (69% GC content) region in the 5' untranslated (UTR) region is underlined. A potential nuclear localization signal is boxed (residues 165-181).

20 Figure 3. The amino acid sequence encoded by the open reading frame of the nucleic acid sequence set forth in Figure 2 (SEQ ID NO: 2).

Figure 4A-4B. Sequence alignment of 55P4H4 with human hypoxia regulated gene products using the BLAST function (NCBI). 4A is a sequence alignment of 55P4H4 with RTP801 (rat isoform) (SEQ ID NO: 5) showing a 32% identity. 4B is a sequence alignment of 55P4H4 with RTP779 (human  
25 isoform) (SEQ ID NO: 6) showing a 32% identity.

Figure 5A-5C. Sequence alignment of 55P4H4 with murine RIK, Drosophila CHARBYE and yeast RIC1 proteins. 5A shows the sequence alignment of 55P4H4 with murine RIK protein (SEQ ID NO: 7), a mouse protein RIK of unknown function (85% identity). 5B shows the sequence alignment of 55P4H4 with Drosophila CHARYBDE protein (SEQ ID NO: 8), with 33% identity and 49%  
30 homology. 5C shows the sequence alignment of 55P4H4 with yeast RIC-1 protein (SEQ ID NO: 9), with 27% identity and 47% homology.

Figure 6A-C. Northern blot analysis of 55P4H4 expression in various normal human tissues (using the 55P4H4 SSH fragment as a probe) and LAPC xenografts. Two multiple tissue northern blots (Clontech) with 2 µg of mRNA/lane, and LAPC xenograft northern blots with 10 µg of total  
35 RNA/lane were probed with the 55P4H4 SSH fragment. Size standards in kilobases (kb) are indicated on the side. For Figure 6A lanes represent: 1) heart; 2) brain; 3) placenta; 4) lung; 5) liver; 6) skeletal

muscle; 7) kidney; 8) pancreas. For Figure 6B lanes represent: 1) spleen; 2) thymus; 3) prostate; 4) testis; 5) ovary; 6) small intestine; 7) colon; 8) leukocytes. For Figure 6C lanes represent: 1) prostate; 2) LAPC-4AD; 3) LAPC-4AI; 4) LAPC-9AD; 5) LAPC-9 AI.

5 Figure 7. Northern blot analysis of 55P4H4 expression in LAPC xenografts that were grown subcutaneously (sc) or intra-tibially (it) within the mouse bone. Northern blots with 10µg of total RNA/lane were probed with the 55P4H4 SSH fragment. Size standards in kilobases (kb) are indicated on the side. Lanes represent: 1) LAPC-4 AD sc; 2) LAPC-4 AD sc; 3) LAPC-4 AD sc; 4) LAPC-4 AD it; 5) LAPC-4 AD it; 6) LAPC-4 AD it.

10 Figure 8. Expression of 55P4H4 in cancer cell lines. RNA was extracted from the LAPC xenograft and a number of cancer cell lines. Northern blots with 10 µg of total RNA/lane were probed with the 55P4H4 SSH fragment. Size standards in kilobases (kb) are indicated on the side. Lanes represent: 1) LAPC-4 AD; 2) LAPC-4 AI; 3) LAPC-9 AD; 4) LAPC-9 AI; 5) TSUPR-1 6) DU145; 7) LNCaP; 8) PC-3; 9) LAPC-4 GL; 10) PrEC; 11) HT1197; 12) SCaBER; 13) UM-UC-3; 14) TCCSUP; 15) J82; 16) 5637; 17) 293T; 18) RD-ES; 19) NCCIT; 20) TERA-1; 21) TERA-2; 22) A431 23) HeLa; 24) OV-1063; 25) PA-1; 26) SW626; 27) CAO V-3; 28) PFSK-1; 29) T98G; 30) SK-ES-1; 31) HOS; 32) U2-OS; 33) RD-ES; 34) CALU-1; 35) A427; 36) NCI-H82; 37) NCI-H146; 38) 769-P; 39) A498; 40) CAKI-1; 41) SW839.

20 Figure 9. Expression of 55P4H4 in prostate cancer patient samples. RNA was extracted from the prostate tumors and their normal adjacent tissue derived from prostate cancer patients. Northern blots with 10 µg of total RNA/lane were probed with the 55P4H4 SSH fragment. Size standards in kilobases (kb) are indicated on the side. Lanes represent: 1) Patient 1, normal adjacent tissue; 2) Patient 1, Gleason 9 tumor; 3) Patient 2, normal adjacent tissue; 4) Patient 2, Gleason 7 tumor; 5) Patient 3, normal adjacent tissue ; 6) Patient 3, Gleason 7 tumor.

25 Figure 10. RT-PCR Expression analysis of 55P4H4. cDNAs generated using tissue pools from multiple cancers were normalized using beta-actin primers and used to study the expression of 55P4H4. Aliquots of the RT-PCR mix after 30 cycles were run on the agarose gel to allow semi-quantitative evaluation of the levels of expression between samples. The first strand cDNAs in the various lanes of this figure are as follows: Lane 1 is a xenograft tissue pool comprised of LAPC4AD, LAPC4AI, LAPC9AD, and LAPC9AI; lane 2 is a prostate cancer tissue pool; lane 3 is from a lung cancer patient; lane 4 is from an ovarian cancer tissue pool; and lane 5 is a water blank. RT-PCR expression was performed on first strand cDNAs generated using pools of tissues from multiple samples. The cDNAs were subsequently normalized using beta-actin PCR. The highest expression was observed in the xenograft pool and in the ovarian cancer tissue pool. Lower levels of expression were also observed in the prostate cancer tissue pool and in the lung cancer tissue pool.

35 Figure 11. Hydrophilicity amino acid profile of 55P5H4 determined by computer algorithm sequence analysis using the method of Hopp and Woods (Hopp T.P., Woods K.R., 1981. Proc. Natl.

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Acad. Sci. U.S.A. 78:3824-3828) accessed on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) through the ExPasy molecular biology server.

Figure 12. Hydropathicity amino acid profile of 55P5H4 determined by computer algorithm sequence analysis using the method of Kyte and Doolittle (Kyte J., Doolittle R.F., 1982. J. Mol. Biol. 157:105-132) accessed on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) through the ExPasy molecular biology server.

Figure 13. Percent accessible residues amino acid profile of 55P5H4 determined by computer algorithm sequence analysis using the method of Janin (Janin J., 1979 Nature 277:491-492) accessed on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) through the ExPasy molecular biology server.

Figure 14. Average flexibility amino acid profile of 55P5H4 determined by computer algorithm sequence analysis using the method of Bhaskaran and Ponnuswamy (Bhaskaran R., and Ponnuswamy P.K., 1988. Int. J. Pept. Protein Res. 32:242-255) accessed on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) through the ExPasy molecular biology server.

Figure 15. Beta-turn amino acid profile of 55P5H4 determined by computer algorithm sequence analysis using the method of Deleage and Roux (Deleage, G., Roux B. 1987 Protein Engineering 1:289-294) accessed on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) through the ExPasy molecular biology server.

Figure 16. Expression of 55P4H4 in human cancer patient specimens. RNA was extracted from human cancer cell lines. Northern blots with 10 µg of total RNA/lane from a pool of 3 patients for each tumor type were generated. Northern blots were probed with the 55P4H4 sequences. Size standards in kilobases (kb) are indicated on the side. Results show expression of 55P4H4 in prostate, kidney, lung and ovary patient tumor pools.

Figure 17. Expression of 55P4H4 in lung cancer samples. RNA was extracted from the lung cancer cell lines CALU-1, A427 and NCI-H82, and from lung tumor (T) and its normal adjacent tissue (NAT) derived from a lung cancer patient diagnosed with squamous cell lung carcinoma grade IIIA. Northern blots with 10 µg of total RNA/lane were probed with the 55P4H4 sequences. Size standards in kilobases (kb) are indicated on the side. Results show expression of 55P4H4 the NCI-H82 cell line and in the patient tumor but not in normal adjacent tissue

## DETAILED DESCRIPTION OF THE INVENTION

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**I.) Definitions:**

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the

art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

As used herein, the terms "advanced prostate cancer", "locally advanced prostate cancer", "advanced disease" and "locally advanced disease" mean prostate cancers that have extended through the prostate capsule, and are meant to include stage C disease under the American Urological Association (AUA) system, stage C1 - C2 disease under the Whitmore-Jewett system, and stage T3 - T4 and N+ disease under the TNM (tumor, node, metastasis) system. In general, surgery is not recommended for patients with locally advanced disease, and these patients have substantially less favorable outcomes compared to patients having clinically localized (organ-confined) prostate cancer. Locally advanced disease is clinically identified by palpable evidence of induration beyond the lateral border of the prostate, or asymmetry or induration above the prostate base. Locally advanced prostate cancer is presently diagnosed pathologically following radical prostatectomy if the tumor invades or penetrates the prostatic capsule, extends into the surgical margin, or invades the seminal vesicles.

"Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence 55P4H4 (either by removing the underlying glycosylation site or by deleting the glycosylation by chemical and/or enzymatic means), and/or adding one or more glycosylation sites that are not present in the native sequence 55P4H4. In addition, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

The term "analog" refers to a molecule which is structurally similar or shares similar or corresponding attributes with another molecule (e.g. a 55P4H4-related protein). For example an analog of the 55P4H4 protein can be specifically bound by an antibody or T cell that specifically binds to 55P4H4.

The term "antibody" is used in the broadest sense. Therefore an "antibody" can be naturally occurring or man-made such as monoclonal antibodies produced by conventional hybridoma technology. Anti-55P4H4 antibodies comprise monoclonal and polyclonal antibodies as well as fragments containing the antigen-binding domain and/or one or more complementarity determining regions of these antibodies.

As used herein, an "antibody fragment" is defined as at least a portion of the variable region of the immunoglobulin molecule that binds to its target, i.e., the antigen-binding region. In one embodiment it specifically covers single anti-55P4H4 antibodies and clones thereof (including agonist, antagonist and neutralizing antibodies) and anti-55P4H4 antibody compositions with polypeptopic specificity.

The term "codon optimized sequences" refers to nucleotide sequences that have been optimized for a particular host species by replacing any codons having a usage frequency of less than about 20%. Nucleotide sequences that have been optimized for expression in a given host species by elimination of spurious polyadenylation sequences, elimination of exon/intron splicing signals, 5 elimination of transposon-like repeats and/or optimization of GC content in addition to codon optimization are referred to herein as an "expression enhanced sequences."

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes 10 chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof. Examples of cytotoxic agents include, but are not limited to maytansinoids, yttrium, bismuth ricin, ricin A-chain, doxorubicin, daunorubicin, taxol, ethidium bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin, diphtheria toxin, *Pseudomonas* exotoxin (PE) A, PE40, abrin, abrin A chain, modeccin A chain, alpha-sarcin, gelonin, mitogellin, 15 retstrictocin, phenomycin, enomycin, curicin, crotin, calicheamicin, sapaonaria officinalis inhibitor, and glucocorticoid and other chemotherapeutic agents, as well as radioisotopes such as  $\text{At}^{211}$ ,  $\text{I}^{131}$ ,  $\text{I}^{125}$ ,  $\text{Y}^{90}$ ,  $\text{Re}^{186}$ ,  $\text{Re}^{188}$ ,  $\text{Sm}^{153}$ ,  $\text{Bi}^{212}$ ,  $\text{P}^{32}$  and radioactive isotopes of Lu. Antibodies may also be conjugated to an anti-cancer pro-drug activating enzyme capable of converting the pro-drug to its active form.

The term "homolog" refers to a molecule which exhibits homology to another molecule, by for 20 example, having sequences of chemical residues that are the same or similar at corresponding positions.

As used herein, the terms "hybridize", "hybridizing", "hybridizes" and the like, used in the context of polynucleotides, are meant to refer to conventional hybridization conditions, preferably such as hybridization in 50% formamide/6XSSC/0.1% SDS/100  $\mu\text{g/ml}$  ssDNA, in which temperatures for hybridization are above 37 degrees C and temperatures for washing in 0.1XSSC/0.1% SDS are above 25 55 degrees C.

As used herein, a polynucleotide is said to be "isolated" when it is substantially separated from contaminant polynucleotides that correspond or are complementary to genes other than the 55P4H4 gene or that encode polypeptides other than 55P4H4 gene product or fragments thereof. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated 55P4H4 polynucleotide.

30 As used herein, a protein is said to be "isolated" when physical, mechanical or chemical methods are employed to remove the 55P4H4 protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated 55P4H4 protein. Alternatively, an isolated protein can be prepared by chemical means.

35 The term "mammal" as used herein refers to any organism classified as a mammal, including mice, rats, rabbits, dogs, cats, cows, horses and humans. In one embodiment of the invention, the mammal is a mouse. In another embodiment of the invention, the mammal is a human.

As used herein, the terms "metastatic prostate cancer" and "metastatic disease" mean prostate cancers that have spread to regional lymph nodes or to distant sites, and are meant to include stage D disease under the AUA system and stage TxNxM+ under the TNM system. As is the case with locally advanced prostate cancer, surgery is generally not indicated for patients with metastatic disease, and hormonal (androgen ablation) therapy is a preferred treatment modality. Patients with metastatic prostate cancer eventually develop an androgen-refractory state within 12 to 18 months of treatment initiation. Approximately half of these androgen-refractory patients die within 6 months after developing that status. The most common site for prostate cancer metastasis is bone. Prostate cancer bone metastases are often osteoblastic rather than osteolytic (i.e., resulting in net bone formation). Bone metastases are found most frequently in the spine, followed by the femur, pelvis, rib cage, skull and humerus. Other common sites for metastasis include lymph nodes, lung, liver and brain. Metastatic prostate cancer is typically diagnosed by open or laparoscopic pelvic lymphadenectomy, whole body radionuclide scans, skeletal radiography, and/or bone-lesion-biopsy.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the antibodies comprising the population are identical except for possible naturally occurring mutations that are present in minor amounts.

As used herein "motif" as in biological motif of an 55P4H4-related protein, refers to any pattern of amino acids forming part of the primary sequence of a protein, that is associated with a particular function (e.g. protein-protein interaction, protein-DNA interaction, etc) or modification (e.g. that is phosphorylated, glycosylated or amidated), or localization (e.g. secretory sequence, nuclear localization sequence, etc.) or a sequence that is correlated with being immunogenic, either humorally or cellularly. A motif can be either contiguous or capable of being aligned to certain positions that are generally correlated with a certain function or property.

As used herein, the term "polynucleotide" means a polymeric form of nucleotides of at least 10 bases or base pairs in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide, and is meant to include single and double stranded forms of DNA and/or RNA. In the art, this term is often used interchangeably with "oligonucleotide". A polynucleotide can comprise a nucleotide sequence disclosed herein wherein thymidine (T) (as shown for example in SEQ ID NO: 1) can also be uracil (U); this definition pertains to the differences between the chemical structures of DNA and RNA, in particular the observation that one of the four major bases in RNA is uracil (U) instead of thymidine (T).

As used herein, the term "polypeptide" means a polymer of at least about 4, 5, 6, 7, or 8 amino acids. Throughout the specification, standard three letter or single letter designations for amino acids are used. In the art, this term is often used interchangeably with "peptide" or "protein".

As used herein, a "recombinant" DNA or RNA molecule is a DNA or RNA molecule that has been subjected to molecular manipulation *in vitro*.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured nucleic acid sequences to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature that can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, are identified by, but not limited to, those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. "Moderately stringent conditions" are described by, but not limited to, those in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

A "transgenic animal" (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A "transgene" is a DNA that is integrated into the genome of a cell from which a transgenic animal develops.

The term "variant" refers to a molecule that exhibits a variation from a described type or norm, such as a protein that has one or more different amino acid residues in the corresponding position(s) of a

specifically described protein (e.g. the 5SP4H4 protein shown in Figure 2). An analog is an example of a variant protein.

As used herein, the 5SP4H4-related gene and 5SP4H4-related protein includes the 5SP4H4 genes and proteins specifically described herein, as well as structurally and/or functionally similar variants or analog of the foregoing. 5SP4H4 peptide analogs generally share at least about 50%, 60%, 70%, 80%, 90% or more amino acid homology (using BLAST criteria). 5SP4H4 nucleotide analogs preferably share 50%, 60%, 70%, 80%, 90% or more nucleic acid homology (using BLAST criteria). In some embodiments, however, lower homology is preferred so as to select preferred residues in view of species-specific codon preferences and/or optimal peptide epitopes tailored to a particular target population, as is appreciated by those skilled in the art.

The 5SP4H4-related proteins of the invention include those specifically identified herein, as well as allelic variants, conservative substitution variants, analogs and homologs that can be isolated/generated and characterized without undue experimentation following the methods outlined herein or readily available in the art. Fusion proteins that combine parts of different 5SP4H4 proteins or fragments thereof, as well as fusion proteins of a 5SP4H4 protein and a heterologous polypeptide are also included. Such 5SP4H4 proteins are collectively referred to as the 5SP4H4-related proteins, the proteins of the invention, or 5SP4H4. As used herein, the term "5SP4H4-related protein" refers to a polypeptide fragment or an 5SP4H4 protein sequence of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more than 25 amino acids; or, at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 85, 90, 95, 100 or more than 100 amino acids.

## **II.) Properties of 5SP4H4.**

As disclosed herein, 5SP4H4 exhibits specific properties that are analogous to those found in a family of molecules whose polynucleotides, polypeptides, reactive cytotoxic T cells (CTL), reactive helper T cells (HTL) and anti-polypeptide antibodies are used in well known diagnostic assays that examine conditions associated with dysregulated cell growth such as cancer, in particular prostate cancer (see, e.g., both its highly specific pattern of tissue expression as well as its overexpression in prostate cancers as described for example in Example 4). The best-known member of this class is PSA, the archetypal marker that has been used by medical practitioners for years to identify and monitor the presence of prostate cancer (see, e.g., Merrill et al., J. Urol. 163(2): 503-5120 (2000); Polascik et al., J. Urol. Aug; 162(2):293-306 (1999) and Fortier et al., J. Nat. Cancer Inst. 91(19): 1635-1640(1999)). A variety of other diagnostic markers are also used in this context including p53 and K-ras (see, e.g., Tulchinsky et al., Int J Mol Med 1999 Jul 4(1):99-102 and Minimoto et al., Cancer Detect Prev 2000;24(1):1-12). Therefore, this disclosure of the 5SP4H4 polynucleotides and polypeptides (as well as the 5SP4H4 polynucleotide probes and anti-5SP4H4 antibodies used to identify the presence of these molecules) and their properties allows skilled artisans to utilize these molecules in methods that are

analogous to those used, for example, in a variety of diagnostic assays directed to examining conditions associated with cancer.

5 Typical embodiments of diagnostic methods which utilize the 55P4H4 polynucleotides, polypeptides, reactive T cells and antibodies are analogous to those methods from well-established diagnostic assays which employ, e.g., PSA polynucleotides, polypeptides, reactive T cells and antibodies. For example, just as PSA polynucleotides are used as probes (for example in Northern analysis, see, e.g., Sharief et al., Biochem. Mol. Biol. Int. 33(3):567-74(1994)) and primers (for example in PCR analysis, see, e.g., Okegawa et al., J. Urol. 163(4): 1189-1190 (2000)) to observe the presence and/or the level of PSA mRNAs in methods of monitoring PSA overexpression or the metastasis of prostate cancers, the 55P4H4 polynucleotides described herein can be utilized in the same way to detect 55P4H4 overexpression or the metastasis of prostate and other cancers expressing this gene. Alternatively, just as PSA polypeptides are used to generate antibodies specific for PSA which can then be used to observe the presence and/or the level of PSA-proteins in methods to monitor PSA protein overexpression (see, e.g., Stephan et al., Urology 55(4):560-3 (2000)) or the metastasis of prostate cells (see, e.g., Alanen et al., Pathol. Res. Pract. 192(3):233-7 (1996)), the 55P4H4 polypeptides described herein can be utilized to generate antibodies for use in detecting 55P4H4 overexpression or the metastasis of prostate cells and cells of other cancers expressing this gene.

Specifically, because metastases involves the movement of cancer cells from an organ of origin (such as the lung or prostate gland etc.) to a different area of the body (such as a lymph node), assays which examine a biological sample for the presence of cells expressing 55P4H4 polynucleotides and/or polypeptides can be used to provide evidence of metastasis. For example, when a biological sample from tissue that does not normally contain 55P4H4-expressing cells (lymph node) is found to contain 55P4H4-expressing cells such as the 55P4H4 expression seen in LAPC4 and LAPC9, xenografts isolated from lymph node and bone metastasis, respectively, this finding is indicative of metastasis.

Alternatively 55P4H4 polynucleotides and/or polypeptides can be used to provide evidence of cancer, for example, when cells in a biological sample that do not normally express 55P4H4 or express 55P4H4 at a different level are found to express 55P4H4 or have an increased expression of 55P4H4 (see, e.g., the 55P4H4 expression in kidney, lung and prostate cancer cells and in patient samples etc. shown in Figures 6-9). In such assays, artisans may further wish to generate supplementary evidence of metastasis by testing the biological sample for the presence of a second tissue restricted marker (in addition to 55P4H4) such as PSA, PSCA etc. (see, e.g., Alanen et al., Pathol. Res. Pract. 192(3): 233-237 (1996)).

Just as PSA polynucleotide fragments and polynucleotide variants are employed by skilled artisans for use in methods of monitoring PSA, 55P4H4 polynucleotide fragments and polynucleotide variants are used in an analogous manner. In particular, typical PSA polynucleotides used in methods



of monitoring PSA are probes or primers which consist of fragments of the PSA cDNA sequence. Illustrating this, primers used to PCR amplify a PSA polynucleotide must include less than the whole PSA sequence to function in the polymerase chain reaction. In the context of such PCR reactions, skilled artisans generally create a variety of different polynucleotide fragments that can be used as primers in order to amplify different portions of a polynucleotide of interest or to optimize amplification reactions (see, e.g., Caetano-Anolles, G. *Biotechniques* 25(3): 472-476, 478-480 (1998); Robertson et al., *Methods Mol. Biol.* 98:121-154 (1998)). An additional illustration of the use of such fragments is provided in Example 4, where a 55P4H4 polynucleotide fragment is used as a probe to show the expression of 55P4H4 RNAs in cancer cells. In addition, variant polynucleotide sequences are typically used as primers and probes for the corresponding mRNAs in PCR and Northern analyses (see, e.g., Sawai et al., *Fetal Diagn. Ther.* 1996 Nov-Dec 11(6):407-13 and *Current Protocols In Molecular Biology*, Volume 2, Unit 2, Frederick M. Ausubel et al. eds., 1995)). Polynucleotide fragments and variants are useful in this context where they are capable of binding to a target polynucleotide sequence (e.g. the 55P4H4 polynucleotide shown in SEQ ID NO: 1) under conditions of high stringency.

Furthermore, PSA polypeptides which contain an epitope that can be recognized by an antibody or T cell that specifically binds to that epitope are used in methods of monitoring PSA. 55P4H4 polypeptide fragments and polypeptide analogs or variants can also be used in an analogous manner. This practice of using polypeptide fragments or polypeptide variants to generate antibodies (such as anti-PSA antibodies or T cells) is typical in the art with a wide variety of systems such as fusion proteins being used by practitioners (see, e.g., *Current Protocols In Molecular Biology*, Volume 2, Unit 16, Frederick M. Ausubel et al. eds., 1995). In this context, each epitope(s) functions to provide the architecture with which an antibody or T cell is reactive. Typically, skilled artisans create a variety of different polypeptide fragments that can be used in order to generate immune responses specific for different portions of a polypeptide of interest (see, e.g., U.S. Patent No. 5,840,501 and U.S. Patent No. 5,939,533). For example it may be preferable to utilize a polypeptide comprising one of the 55P4H4 biological motifs discussed herein or available in the art. Polypeptide fragments, variants or analogs are typically useful in this context as long as they comprise an epitope capable of generating an antibody or T cell specific for a target polypeptide sequence (e.g. the 55P4H4 polypeptide shown in SEQ ID NO: 2).

As shown herein, the 55P4H4 polynucleotides and polypeptides (as well as the 55P4H4 polynucleotide probes and anti-55P4H4 antibodies or T cells used to identify the presence of these molecules) exhibit specific properties that make them useful in diagnosing cancers of the prostate. Diagnostic assays that measure the presence of 55P4H4 gene products, in order to evaluate the presence or onset of a disease condition described herein, such as prostate cancer, are used to identify patients for preventive measures or further monitoring, as has been done so successfully with PSA.

Moreover, these materials satisfy a need in the art for molecules having similar or complementary characteristics to PSA in situations where, for example, a definite diagnosis of metastasis of prostatic origin cannot be made on the basis of a test for PSA alone (see, e.g., Alanen et al., Pathol. Res. Pract. 192(3): 233-237 (1996)), and consequently, materials such as 55P4H4 polynucleotides and polypeptides (as well as the 55P4H4 polynucleotide probes and anti-55P4H4 antibodies used to identify the presence of these molecules) must be employed to confirm metastases of prostatic origin.

Finally, in addition to their use in diagnostic assays, the 55P4H4 polynucleotides disclosed herein have a number of other specific utilities such as their use in the identification of oncogenic associated chromosomal abnormalities in the chromosomal region to which the 55P4H4 gene maps (see Example 3 below). Moreover, in addition to their use in diagnostic assays, the 55P4H4-related proteins and polynucleotides disclosed herein have other utilities such as their use in the forensic analysis of tissues of unknown origin (see, e.g., Takahama K Forensic Sci Int 1996 Jun 28;80(1-2): 63-9).

Additionally, 55P4H4-related proteins or polynucleotides of the invention can be used to treat a pathologic condition characterized by the over-expression of 55P4H4. For example, the amino acid or nucleic acid sequence of Figure 2, or fragments thereof, can be used to generate an immune response to the 55P4H4 antigen. Antibodies or other molecules that react with 55P4H4 can be used to modulate the function of this molecule, and thereby provide a therapeutic benefit.

### III.) 55P4H4 Polynucleotides

One aspect of the invention provides polynucleotides corresponding or complementary to all or part of an 55P4H4 gene, mRNA, and/or coding sequence, preferably in isolated form, including polynucleotides encoding an 55P4H4-related protein and fragments thereof, DNA, RNA, DNA/RNA hybrid, and related molecules, polynucleotides or oligonucleotides complementary to an 55P4H4 gene or mRNA sequence or a part thereof, and polynucleotides or oligonucleotides that hybridize to an 55P4H4 gene, mRNA, or to an 55P4H4 encoding polynucleotide (collectively, "55P4H4 polynucleotides"). In all instances when referred to in this section, T can also be U in Figure 2.

Embodiments of a 55P4H4 polynucleotide include: a 55P4H4 polynucleotide having the sequence shown in Figure 2, the nucleotide sequence of 55P4H4 as shown in Figure 2, wherein T is U; at least 10 contiguous nucleotides of a polynucleotide having the sequence as shown in Figure 2; or, at least 10 contiguous nucleotides of a polynucleotide having the sequence as shown in Figure 2 where T is U. Embodiments of 55P4H4 nucleotides comprise, without limitation:

- (a) a polynucleotide consisting of the sequence as shown in SEQ ID NO: 1, wherein T can also be U;

(b) a polynucleotide consisting of the sequence as shown in SEQ ID NO: 1, from nucleotide residue number 204 through nucleotide residue number 785, wherein T can also be U;

5 (c) a polynucleotide that encodes a 55P4H4-related protein whose sequence is encoded by the cDNAs contained in the plasmid designated p55P4H4-EBV12 deposited with American Type Culture Collection as Accession No. PTA-1894;

(d) a polynucleotide that encodes an 55P4H4-related protein that is at least 90% homologous to the entire amino acid sequence shown in SEQ ID NO: 2;

10 (e) a polynucleotide that encodes an 55P4H4-related protein that is at least 90% identical to the entire amino acid sequence shown in SEQ ID NO: 2;

(f) a polynucleotide that encodes at least one peptide set forth in Tables V-XVIII;

(g) a polynucleotide that encodes a peptide region of at least 5 amino acids of Figure 3 in any whole number increment up to 193 that includes an amino acid position having a value greater than 0.5 in the Hydrophilicity profile of Figure 11;

15 (h) a polynucleotide that encodes a peptide region of at least 5 amino acids of Figure 3 in any whole number increment up to 193 that includes an amino acid position having a value less than 0.5 in the Hydropathicity profile of Figure 12;

20 (I) a polynucleotide that encodes a peptide region of at least 5 amino acids of Figure 3 in any whole number increment up to 193 that includes an amino acid position having a value greater than 0.5 in the Percentage Accessible Residues profile of Figure 13;

(j) a polynucleotide that encodes a peptide region of at least 5 amino acids of Figure 3 in any whole number increment up to 193 that includes an amino acid position having a value greater than 0.5 in the Average Flexibility profile on Figure 14;

25 (k) a polynucleotide that encodes a peptide region of at least 5 amino acids of Figure 3 in any whole number increment up to 193 that includes an amino acid position having a value greater than 0.5 in the Beta-turn profile of Figure 15;

(l) a polynucleotide that is fully complementary to a polynucleotide of any one of (a)-(k);

- (m) a polynucleotide that selectively hybridizes under stringent conditions to a polynucleotide of (a)-(l);
- (n) a peptide that is encoded by any of (a)-(k); and,
- (o) a polynucleotide of any of (a)-(m) together with a pharmaceutical excipient and/or in a human unit dose form;
- (p) a peptide of (n) together with a pharmaceutical excipient and/or in a human unit dose form;

wherein a range as used herein is understood to specifically disclose all whole unit positions thereof.

An alternative embodiment comprises a polynucleotide or protein/peptide of the invention together with a pharmaceutical excipient and/or in a human unit dose form.

Typical embodiments of the invention disclosed herein include 55P4H4 polynucleotides that encode specific portions of the 55P4H4 mRNA sequence (and those which are complementary to such sequences) such as those that encode the protein and fragments thereof, for example of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or 193 contiguous amino acids.

For example, representative embodiments of the invention disclosed herein include: polynucleotides and their encoded peptides themselves encoding about amino acid 1 to about amino acid 10 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 10 to about amino acid 20 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 20 to about amino acid 30 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 30 to about amino acid 40 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 40 to about amino acid 50 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 50 to about amino acid 60 of the 55P4H4 protein shown in Figure 2 or Figure 3, polynucleotides encoding about amino acid 60 to about amino acid 70 of the 55P4H4 protein shown in Figure 2 or Figure 3, in increments of about 10 amino acids, ending at the carboxyl terminal amino acid set forth in Figure 2 or Figure 3. Accordingly polynucleotides encoding portions of the amino acid sequence (of about 10 amino acids), of amino acids 70 through the carboxyl terminal amino acid of the 55P4H4 protein are embodiments of the invention. Wherein it is understood that each particular amino acid position discloses that specific position plus or minus five contiguous amino acid residues.

Polynucleotides encoding relatively long portions of the 55P4H4 protein are also within the scope of the invention. For example, polynucleotides encoding from about amino acid 1 (or 20 or 30 or 40 etc.) to about amino acid 20, (or 30, or 40 or 50 etc.) of the 55P4H4 protein shown in Figure 2 can be generated by a variety of techniques well known in the art. These polynucleotide fragments can include any portion of the 55P4H4 sequence as shown in Figure 2.

Additional illustrative embodiments of the invention disclosed herein include 55P4H4 polynucleotide fragments encoding one or more of the biological motifs contained within the 55P4H4 protein sequence, including one or more of the motif-bearing subsequences of the 55P4H4 protein set forth in Tables V-XIX. In another embodiment, typical polynucleotide fragments of the invention  
5 encode one or more of the regions of 55P4H4 that exhibit homology to a known molecule. In another embodiment of the invention, typical polynucleotide fragments can encode one or more of the 55P4H4 biological motifs disclosed herein including the N-glycosylation site, the protein kinase C phosphorylation sites, the casein kinase II phosphorylation sites or the N-myristoylation sites.

### 10 III.A.) Uses of 55P4H4 Polynucleotides

#### III.A.1.) Monitoring of Genetic Abnormalities

The polynucleotides of the preceding paragraphs have a number of different specific uses. The human 55P4H4 gene maps to the chromosomal location set forth in Example 3. For example, because the 55P4H4 gene maps to this chromosome, polynucleotides that encode different regions of  
15 the 55P4H4 protein are used to characterize cytogenetic abnormalities of this chromosomal locale, such as abnormalities that are identified as being associated with various cancers. In certain genes, a variety of chromosomal abnormalities including rearrangements have been identified as frequent cytogenetic abnormalities in a number of different cancers (see e.g. Krajinovic et al., Mutat. Res. 382(3-4): 81-83 (1998); Johansson et al., Blood 86(10): 3905-3914 (1995) and Finger et al., P.N.A.S. 85(23): 9158-  
20 9162 (1988)). Thus, polynucleotides encoding specific regions of the 55P4H4 protein provide new tools that can be used to delineate, with greater precision than previously possible, cytogenetic abnormalities in the chromosomal region that encodes 55P4H4 that may contribute to the malignant phenotype. In this context, these polynucleotides satisfy a need in the art for expanding the sensitivity of chromosomal screening in order to identify more subtle and less common chromosomal  
25 abnormalities (see e.g. Evans et al., Am. J. Obstet. Gynecol 171(4): 1055-1057 (1994)).

Furthermore, as 55P4H4 was shown to be highly expressed in prostate and other cancers, 55P4H4 polynucleotides are used in methods assessing the status of 55P4H4 gene products in normal versus cancerous tissues. Typically, polynucleotides that encode specific regions of the 55P4H4 protein are used to assess the presence of perturbations (such as deletions, insertions, point mutations,  
30 or alterations resulting in a loss of an antigen etc.) in specific regions of the 55P4H4 gene, such as such regions containing one or more motifs. Exemplary assays include both RT-PCR assays as well as single-strand conformation polymorphism (SSCP) analysis (see, e.g., Marrogi et al., J. Cutan. Pathol. 26(8): 369-378 (1999), both of which utilize polynucleotides encoding specific regions of a protein to examine these regions within the protein.

### III.A.2.) Antisense Embodiments

Other specifically contemplated nucleic acid related embodiments of the invention disclosed herein are genomic DNA, cDNAs, ribozymes, and antisense molecules, as well as nucleic acid molecules based on an alternative backbone, or including alternative bases, whether derived from natural sources or synthesized, and include molecules capable of inhibiting the RNA or protein expression of 55P4H4. For example, antisense molecules can be RNAs or other molecules, including peptide nucleic acids (PNAs) or non-nucleic acid molecules such as phosphorothioate derivatives, that specifically bind DNA or RNA in a base pair-dependent manner. A skilled artisan can readily obtain these classes of nucleic acid molecules using the 55P4H4 polynucleotides and polynucleotide sequences disclosed herein.

Antisense technology entails the administration of exogenous oligonucleotides that bind to a target polynucleotide located within the cells. The term "antisense" refers to the fact that such oligonucleotides are complementary to their intracellular targets, e.g., 55P4H4. See for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press, 1989; and *Synthesis* 1:1-5 (1988). The 55P4H4 antisense oligonucleotides of the present invention include derivatives such as S-oligonucleotides (phosphorothioate derivatives or S-oligos, see, Jack Cohen, supra), which exhibit enhanced cancer cell growth inhibitory action. S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligo) in which a nonbridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention can be prepared by treatment of the corresponding O-oligos with 3H-1,2-benzodithiol-3-one-1,1-dioxide, which is a sulfur transfer reagent. See Iyer, R. P. et al, *J. Org. Chem.* 55:4693-4698 (1990); and Iyer, R. P. et al., *J. Am. Chem. Soc.* 112:1253-1254 (1990). Additional 55P4H4 antisense oligonucleotides of the present invention include morpholino antisense oligonucleotides known in the art (see, e.g., Partridge et al., 1996, *Antisense & Nucleic Acid Drug Development* 6: 169-175).

The 55P4H4 antisense oligonucleotides of the present invention typically can be RNA or DNA that is complementary to and stably hybridizes with the first 100 5' codons or last 100 3' codons of the 55P4H4 genomic sequence or the corresponding mRNA. Absolute complementarity is not required, although high degrees of complementarity are preferred. Use of an oligonucleotide complementary to this region allows for the selective hybridization to 55P4H4 mRNA and not to mRNA specifying other regulatory subunits of protein kinase. In one embodiment, 55P4H4 antisense oligonucleotides of the present invention are 15 to 30-mer fragments of the antisense DNA molecule that have a sequence that hybridizes to 55P4H4 mRNA. Optionally, 55P4H4 antisense oligonucleotide is a 30-mer oligonucleotide that is complementary to a region in the first 10 5' codons or last 10 3' codons of 55P4H4. Alternatively, the antisense molecules are modified to employ ribozymes in the inhibition of 55P4H4 expression, see, e.g., L. A. Couture & D. T. Stinchcomb; *Trends Genet* 12: 510-515 (1996).

### **III.A.3.) Primers and Primer Pairs**

Further specific embodiments of this nucleotides of the invention include primers and primer pairs, which allow the specific amplification of polynucleotides of the invention or of any specific parts thereof, and probes that selectively or specifically hybridize to nucleic acid molecules of the invention or to any part thereof. Probes can be labeled with a detectable marker, such as, for example, a radioisotope, fluorescent compound, bioluminescent compound, a chemiluminescent compound, metal chelator or enzyme. Such probes and primers are used to detect the presence of a 55P4H4 polynucleotide in a sample and as a means for detecting a cell expressing a 55P4H4 protein.

Examples of such probes include polypeptides comprising all or part of the human 55P4H4 cDNA sequences shown in Figure 2. Examples of primer pairs capable of specifically amplifying 55P4H4 mRNAs are also described in the Examples. As will be understood by the skilled artisan, a great many different primers and probes can be prepared based on the sequences provided herein and used effectively to amplify and/or detect a 55P4H4 mRNA.

The 55P4H4 polynucleotides of the invention are useful for a variety of purposes, including but not limited to their use as probes and primers for the amplification and/or detection of the 55P4H4 gene(s), mRNA(s), or fragments thereof; as reagents for the diagnosis and/or prognosis of prostate cancer and other cancers; as coding sequences capable of directing the expression of 55P4H4 polypeptides; as tools for modulating or inhibiting the expression of the 55P4H4 gene(s) and/or translation of the 55P4H4 transcript(s); and as therapeutic agents.

### **III.A.4.) Isolation of 55P4H4-Encoding Nucleic Acid Molecules**

The 55P4H4 cDNA sequences described herein enable the isolation of other polynucleotides encoding 55P4H4 gene product(s), as well as the isolation of polynucleotides encoding 55P4H4 gene product homologs, alternatively spliced isoforms, allelic variants, and mutant forms of the 55P4H4 gene product as well as polynucleotides that encode analogs of 55P4H4-related proteins. Various molecular cloning methods that can be employed to isolate full length cDNAs encoding an 55P4H4 gene are well known (see, for example, Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2d edition, Cold Spring Harbor Press, New York, 1989; Current Protocols in Molecular Biology. Ausubel et al., Eds., Wiley and Sons, 1995). For example, lambda phage cloning methodologies can be conveniently employed, using commercially available cloning systems (e.g., Lambda ZAP Express, Stratagene). Phage clones containing 55P4H4 gene cDNAs can be identified by probing with a labeled 55P4H4 cDNA or a fragment thereof. For example, in one embodiment, the 55P4H4 cDNA (Figure 2) or a portion thereof can be synthesized and used as a probe to retrieve overlapping and full-length cDNAs corresponding to a 55P4H4 gene. The 55P4H4 gene itself can be isolated by screening genomic DNA libraries, bacterial artificial chromosome libraries (BACs), yeast artificial chromosome libraries (YACs), and the like, with 55P4H4 DNA probes or primers.

### III.A.5.) Recombinant Nucleic Acid Molecules and Host-Vector Systems

The invention also provides recombinant DNA or RNA molecules containing an 55P4H4 polynucleotide, fragment, analog or homologue thereof, including but not limited to phages, plasmids, phagemids, cosmids, YACs, BACs, as well as various viral and non-viral vectors well known in the art, and cells transformed or transfected with such recombinant DNA or RNA molecules. Methods for generating such molecules are well known (see, for example, Sambrook et al, 1989, supra).

The invention further provides a host-vector system comprising a recombinant DNA molecule containing a 55P4H4 polynucleotide, fragment, analog or homologue thereof within a suitable prokaryotic or eukaryotic host cell. Examples of suitable eukaryotic host cells include a yeast cell, a plant cell, or an animal cell, such as a mammalian cell or an insect cell (e.g., a baculovirus-infectible cell such as an Sf9 or HighFive cell). Examples of suitable mammalian cells include various prostate cancer cell lines such as DU145 and TsuPr1, other transfectable or transducible prostate cancer cell lines, primary cells (PrEC), as well as a number of mammalian cells routinely used for the expression of recombinant proteins (e.g., COS, CHO, 293, 293T cells). More particularly, a polynucleotide comprising the coding sequence of 55P4H4 or a fragment, analog or homolog thereof can be used to generate 55P4H4 proteins or fragments thereof using any number of host-vector systems routinely used and widely known in the art.

A wide range of host-vector systems suitable for the expression of 55P4H4 proteins or fragments thereof are available, see for example, Sambrook et al., 1989, supra; Current Protocols in Molecular Biology, 1995, supra). Preferred vectors for mammalian expression include but are not limited to pcDNA 3.1 myc-His-tag (Invitrogen) and the retroviral vector pSR $\alpha$ tkneo (Muller et al., 1991, MCB 11:1785). Using these expression vectors, 55P4H4 can be expressed in several prostate cancer and non-prostate cell lines, including for example 293, 293T, rat-1, NIH 3T3 and TsuPr1. The host-vector systems of the invention are useful for the production of a 55P4H4 protein or fragment thereof. Such host-vector systems can be employed to study the functional properties of 55P4H4 and 55P4H4 mutations or analogs.

Recombinant human 55P4H4 protein or an analog or homolog or fragment thereof can be produced by mammalian cells transfected with a construct encoding a 55P4H4-related nucleotide. For example, 293T cells can be transfected with an expression plasmid encoding 55P4H4 or fragment, analog or homolog thereof, the 55P4H4 or related protein is expressed in the 293T cells, and the recombinant 55P4H4 protein is isolated using standard purification methods (e.g., affinity purification using anti-55P4H4 antibodies). In another embodiment, a 55P4H4 coding sequence is subcloned into the retroviral vector pSR $\alpha$ MSVtkneo and used to infect various mammalian cell lines, such as NIH 3T3, TsuPr1, 293 and rat-1 in order to establish 55P4H4 expressing cell lines. Various other expression systems well known in the art can also be employed. Expression constructs encoding a



leader peptide joined in frame to the 55P4H4 coding sequence can be used for the generation of a secreted form of recombinant 55P4H4 protein.

As discussed herein, redundancy in the genetic code permits variation in 55P4H4 gene sequences. In particular, it is known in the art that specific host species often have specific codon preferences, and thus one can adapt the disclosed sequence as preferred for a desired host. For example, preferred analog codon sequences typically have rare codons (i.e., codons having a usage frequency of less than about 20% in known sequences of the desired host) replaced with higher frequency codons. Codon preferences for a specific species are calculated, for example, by utilizing codon usage tables available on the INTERNET such as:

<http://www.dna.affrc.go.jp/~nakamura/codon.html>. Additional sequence modifications are known to enhance protein expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon/intron splice site signals, transposon-like repeats, and/or other such well-characterized sequences that are deleterious to gene expression. The GC content of the sequence is adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. Where possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures. Other useful modifications include the addition of a translational initiation consensus sequence at the start of the open reading frame, as described in Kozak, *Mol. Cell Biol.*, 9:5073-5080 (1989). Skilled artisans understand that the general rule that eukaryotic ribosomes initiate translation exclusively at the 5' proximal AUG codon is abrogated only under rare conditions (see, e.g., Kozak PNAS 92(7): 2662-2666, (1995) and Kozak NAR 15(20): 8125-8148 (1987)).

#### IV.) 55P4H4-related Proteins

Another aspect of the present invention provides 55P4H4-related proteins. Specific embodiments of 55P4H4 proteins comprise a polypeptide having all or part of the amino acid sequence of human 55P4H4 as shown in Figure 2. Alternatively, embodiments of 55P4H4 proteins comprise variant, homolog or analog polypeptides that have alterations in the amino acid sequence of 55P4H4 shown in Figure 2.

In general, naturally occurring allelic variants of human 55P4H4 share a high degree of structural identity and homology (e.g., 90% or more homology). Typically, allelic variants of the 55P4H4 protein contain conservative amino acid substitutions within the 55P4H4 sequences described herein or contain a substitution of an amino acid from a corresponding position in a homologue of 55P4H4. One class of 55P4H4 allelic variants are proteins that share a high degree of homology with at least a small region of a particular 55P4H4 amino acid sequence, but further contain a radical departure from the sequence, such as a non-conservative substitution, truncation, insertion or frame shift. In comparisons of protein sequences, the terms, similarity, identity, and homology each have a distinct meaning as appreciated in the field of

genetics. Moreover, orthology and paralogy can be important concepts describing the relationship of members of a given protein family in one organism to the members of the same family in other organisms.

Amino acid abbreviations are provided in Table II. Conservative amino acid substitutions can frequently be made in a protein without altering either the conformation or the function of the protein.

5 Proteins of the invention can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 conservative substitutions. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. Other  
10 substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine (A) and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and  
15 sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments (see, e.g. Table III herein; pages 13-15 "Biochemistry" 2<sup>nd</sup> ED. Lubert Stryer  
ed (Stanford University); Henikoff et al., PNAS 1992 Vol 89 10915-10919; Lei et al., J Biol Chem 1995 May 19; 270(20):11882-6).

Embodiments of the invention disclosed herein include a wide variety of art-accepted variants  
20 or analogs of 55P4H4 proteins such as polypeptides having amino acid insertions, deletions and substitutions. 55P4H4 variants can be made using methods known in the art such as site-directed mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter et al., *Nucl. Acids Res.*, 13:4331 (1986); Zoller et al., *Nucl. Acids Res.*, 10:6487 (1987)), cassette mutagenesis (Wells et al., *Gene*, 34:315 (1985)), restriction selection mutagenesis (Wells et al., *Philos. Trans. R. Soc. London SerA*, 317:415 (1986)) or other known techniques can be performed on the cloned DNA to  
25 produce the 55P4H4 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence that is involved in a specific biological activity such as a protein-protein interaction. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such  
30 amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions (Creighton, *The Proteins*, (W.H. Freeman & Co., N.Y.); Chothia, *J. Mol. Biol.*, 150:1 (1976)). If  
35 alanine substitution does not yield adequate amounts of variant, an isosteric amino acid can be used.

As defined herein, 55P4H4 variants, analogs or homologs, have the distinguishing attribute of

having at least one epitope that is "cross reactive" with a 55P4H4 protein having the amino acid sequence of SEQ ID NO: 2. As used in this sentence, "cross reactive" means that an antibody or T cell that specifically binds to an 55P4H4 variant also specifically binds to the 55P4H4 protein having the amino acid sequence of SEQ ID NO: 2. A polypeptide ceases to be a variant of the protein shown in  
5 SEQ ID NO: 2 when it no longer contains any epitope capable of being recognized by an antibody or T cell that specifically binds to the 55P4H4 protein. Those skilled in the art understand that antibodies that recognize proteins bind to epitopes of varying size, and a grouping of the order of about four or five amino acids, contiguous or not, is regarded as a typical number of amino acids in a minimal epitope. See, e.g., Nair et al., J. Immunol 2000 165(12): 6949-6955; Hebbes et al., Mol Immunol  
10 (1989) 26(9):865-73; Schwartz et al., J Immunol (1985) 135(4):2598-608.

Another class of 55P4H4-related protein variants share 70%, 75%, 80%, 85% or 90% or more similarity with the amino acid sequence of SEQ ID NO: 2 or a fragment thereof. Another specific class of 55P4H4 protein variants or analogs comprise one or more of the 55P4H4-biological motifs described herein or presently known in the art. Thus, encompassed by the present invention are analogs of  
15 55P4H4 fragments (nucleic or amino acid) that have altered functional (e.g. immunogenic) properties relative to the starting fragment. It is to be appreciated that motifs now or which become part of the art are to be applied to the nucleic or amino acid sequences of Figure 2.

As discussed herein, embodiments of the claimed invention include polypeptides containing less than the 532 amino acid sequence of the 55P4H4 protein shown in Figure 2. For example,  
20 representative embodiments of the invention comprise peptides/proteins having any 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more contiguous amino acids of the 55P4H4 protein shown in Figure 2 (SEQ ID NO: 2).

Moreover, representative embodiments of the invention disclosed herein include polypeptides consisting of about amino acid 1 to about amino acid 10 of the 55P4H4 protein shown in Figure 2 or  
25 Figure 3, polypeptides consisting of about amino acid 10 to about amino acid 20 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 20 to about amino acid 30 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 30 to about amino acid 40 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 40 to about amino acid 50 of the 55P4H4 protein shown in Figure 2 or Figure 3,  
30 polypeptides consisting of about amino acid 50 to about amino acid 60 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 60 to about amino acid 70 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 70 to about amino acid 80 of the 55P4H4 protein shown in Figure 2 or Figure 3, polypeptides consisting of about amino acid 80 to about amino acid 90 of the 55P4H4 protein shown in Figure 2 or Figure 3,  
35 polypeptides consisting of about amino acid 90 to about amino acid 100 of the 55P4H4 protein shown in Figure 2 or Figure 3, etc. throughout the entirety of the 55P4H4 amino acid sequence. Moreover,

polypeptides consisting of about amino acid 1 (or 20 or 30 or 40 etc.) to about amino acid 20, (or 130, or 140 or 150 etc.) of the 55P4H4 protein shown in Figure 2 are embodiments of the invention. It is to be appreciated that the starting and stopping positions in this paragraph refer to the specified position as well as that position plus or minus 5 residues.

5 55P4H4-related proteins are generated using standard peptide synthesis technology or using chemical cleavage methods well known in the art. Alternatively, recombinant methods can be used to generate nucleic acid molecules that encode a 55P4H4-related protein. In one embodiment, nucleic acid molecules provide a means to generate defined fragments of the 55P4H4 protein (or variants, homologs or analogs thereof).

#### 10 **IV.A.) Motif-bearing Protein Embodiments**

Additional illustrative embodiments of the invention disclosed herein include 55P4H4 polypeptides comprising the amino acid residues of one or more of the biological motifs contained within the 55P4H4 polypeptide sequence set forth in Figure 2 or Figure 3. Various motifs are known in the art, and a protein can be evaluated for the presence of such motifs by a number of publicly available sites (see, e.g.: <http://pfam.wustl.edu/>; <http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html>; <http://psort.ims.u-tokyo.ac.jp/>; <http://www.cbs.dtu.dk/>; <http://www.ebi.ac.uk/interpro/scan.html>; <http://www.expasy.ch/tools/scnpsit1.html>; Epimatrix™ and Epimer™, Brown University, [http://www.brown.edu/Research/TB-HIV\\_Lab/epimatrix/epimatrix.html](http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html); and BIMAS, <http://bimas.dcrt.nih.gov/>).

20 Motif bearing subsequences of the 55P4H4 protein are set forth and identified in Table XIX.

Table XX sets forth several frequently occurring motifs based on pfam searches (<http://pfam.wustl.edu/>). The columns of Table XX list (1) motif name abbreviation, (2) percent identity found amongst the different member of the motif family, (3) motif name or description and (4) most common function; location information is included if the motif is relevant for location.

25 Polypeptides comprising one or more of the 55P4H4 motifs discussed above are useful in elucidating the specific characteristics of a malignant phenotype in view of the observation that the 55P4H4 motifs discussed above are associated with growth dysregulation and because 55P4H4 is overexpressed in certain cancers (See, e.g., Table I). Casein kinase II, cAMP and camp-dependent protein kinase, and Protein Kinase C, for example, are enzymes known to be associated with the development of the malignant phenotype (see e.g. Chen et al., Lab Invest., 78(2): 165-174 (1998); Gaiddon et al., Endocrinology 136(10): 4331-4338 (1995); Hall et al., Nucleic Acids Research 24(6): 1119-1126 (1996); Peterziel et al., Oncogene 18(46): 6322-6329 (1999) and O'Brian, Oncol. Rep. 5(2): 305-309 (1998)). Moreover, both glycosylation and myristoylation are protein modifications also associated with cancer and cancer progression (see e.g. Dennis et al., Biochem. Biophys. Acta 1473(1):21-34 (1999); Raju et al., Exp. Cell Res. 235(1): 145-154 (1997)). Amidation is another protein modification also associated with cancer and cancer progression (see e.g. Treston et al., J. Natl.

Cancer Inst. Monogr. (13): 169-175 (1992)).

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5 In another embodiment, proteins of the invention comprise one or more of the immunoreactive epitopes identified in accordance with art-accepted methods, such as the peptides set forth in Tables V-XVIII. CTL epitopes can be determined using specific algorithms to identify peptides within an 55P4H4 protein that are capable of optimally binding to specified HLA alleles (e.g., Table IV (A) and Table IV (B); Epimatrix™ and Epimer™, Brown University, [http://www.brown.edu/Research/TB-HIV\\_Lab/epimatrix/epimatrix.html](http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html); and BIMAS, <http://bimas.dcrt.nih.gov/>. Moreover, processes for identifying peptides that have sufficient binding affinity for HLA molecules and which are correlated with being immunogenic epitopes, are well known in the art, and are carried out without undue experimentation. In addition, processes for identifying peptides that are immunogenic epitopes, are well known in the art, and are carried out without undue experimentation either *in vitro* or *in vivo*.

10 Also known in the art are principles for creating analogs of such epitopes in order to modulate immunogenicity. For example, one begins with an epitope that bears a CTL or HTL motif (see, e.g., the HLA Class I motifs of Table IV (A) and the HTL motif of Table IV (B)). The epitope is analoged by substituting out an amino acid at one of the specified positions, and replacing it with another amino acid specified for that position.

15 A variety of references reflect the art regarding the identification and generation of epitopes in a protein of interest as well as analogs thereof. See, for example, WO 9733602 to Chesnut et al.; Sette, Immunogenetics 1999 50(3-4): 201-212; Sette et al., J. Immunol. 2001 166(2): 1389-1397; Sidney et al., Hum. Immunol. 1997 58(1): 12-20; Kondo et al., Immunogenetics 1997 45(4): 249-258; Sidney et al., J. Immunol. 1996 157(8): 3480-90; and Falk et al., Nature 351: 290-6 (1991); Hunt et al., Science 255:1261-3 (1992); Parker et al., J. Immunol. 149:3580-7 (1992); Parker et al., J. Immunol. 152:163-75 (1994); Kast et al., 1994 152(8): 3904-12; Borrás-Cuesta et al., Hum. Immunol. 2000 61(3): 266-278; Alexander et al., J. Immunol. 2000 164(3): 1625-1633; Alexander et al., PMID: 7895164, UI: 95202582; O'Sullivan et al., J. Immunol. 1991 147(8): 2663-2669; Alexander et al., Immunity 1994 1(9): 751-761 and Alexander et al., Immunol. Res. 1998 18(2): 79-92.

20 Related embodiments of the inventions include polypeptides comprising combinations of the different motifs set forth in Table XIX, and/or, one or more of the predicted CTL epitopes of Table V through Table XVIII, and/or, one or more of the T cell binding motifs known in the art. Preferred  
30 embodiments contain no insertions, deletions or substitutions either within the motifs or the intervening sequences of the polypeptides. In addition, embodiments which include a number of either N-terminal and/or C-terminal amino acid residues on either side of these motifs may be desirable (to, for example, include a greater portion of the polypeptide architecture in which the motif is located). Typically the number of N-terminal and/or C-terminal amino acid residues on either side of a motif is between about  
35 1 to about 100 amino acid residues, preferably 5 to about 50 amino acid residues.

55P4H4-related proteins are embodied in many forms, preferably in isolated form. A purified 55P4H4 protein molecule will be substantially free of other proteins or molecules that impair the binding of 55P4H4 to antibody, T cell or other ligand. The nature and degree of isolation and purification will depend on the intended use. Embodiments of a 55P4H4-related proteins include

5 purified 55P4H4-related proteins and functional, soluble 55P4H4-related proteins. In one embodiment, a functional, soluble 55P4H4 protein or fragment thereof retains the ability to be bound by antibody, T cell or other ligand.

The invention also provides 55P4H4 proteins comprising biologically active fragments of the 55P4H4 amino acid sequence shown in Figure 2. Such proteins exhibit properties of the 55P4H4

10 protein, such as the ability to elicit the generation of antibodies that specifically bind an epitope associated with the 55P4H4 protein; to be bound by such antibodies; to elicit the activation of HTL or CTL; and/or, to be recognized by HTL or CTL.

55P4H4-related polypeptides that contain particularly interesting structures can be predicted and/or identified using various analytical techniques well known in the art, including, for example, the

15 methods of Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis, or on the basis of immunogenicity. Fragments that contain such structures are particularly useful in generating subunit-specific anti-55P4H4 antibodies, or T cells or in identifying cellular factors that bind to 55P4H4.

CTL epitopes can be determined using specific algorithms to identify peptides within an 55P4H4

20 protein that are capable of optimally binding to specified HLA alleles (e.g., Table IV (A) and Table IV (B); Epimatrix™ and Epimer™, Brown University ([http://www.brown.edu/Research/TB-HIV\\_Lab/epimatrix/epimatrix.html](http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html)); and BIMAS, <http://bimas.dcrn.nih.gov/>). Illustrating this, peptide epitopes from 55P4H4 that are presented in the context of human MHC class I molecules HLA-A1, A2, A3, A11, A24, B7 and B35 were predicted (Tables V-XVIII). Specifically, the complete amino acid

25 sequence of the 55P4H4 protein was entered into the HLA Peptide Motif Search algorithm found in the Bioinformatics and Molecular Analysis Section (BIMAS) web site listed above. The HLA peptide motif search algorithm was developed by Dr. Ken Parker based on binding of specific peptide sequences in the groove of HLA Class I molecules and specifically HLA-A2 (see, e.g., Falk et al., Nature 351: 290-6 (1991); Hunt et al., Science 255:1261-3 (1992); Parker et al., J. Immunol. 149:3580-

30 7 (1992); Parker et al., J. Immunol. 152:163-75 (1994)). This algorithm allows location and ranking of 8-mer, 9-mer, and 10-mer peptides from a complete protein sequence for predicted binding to HLA-A2 as well as numerous other HLA Class I molecules. Many HLA class I binding peptides are 8-, 9-, 10 or 11-mers. For example, for class I HLA-A2, the epitopes preferably contain a leucine (L) or methionine (M) at position 2 and a valine (V) or leucine (L) at the C-terminus (see, e.g., Parker et al., J. Immunol. 149:3580-7 (1992)).

35 Selected results of 55P4H4 predicted binding peptides are shown in Tables V-XVIII herein. In Tables V-XVIII, the top 50 ranking candidates, 9-mers and 10-mers, for each family

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member are shown along with their location, the amino acid sequence of each specific peptide, and an estimated binding score. The binding score corresponds to the estimated half-time of dissociation of complexes containing the peptide at 37°C at pH 6.5. Peptides with the highest binding score are predicted to be the most tightly bound to HLA Class I on the cell surface for the greatest period of time and thus represent the best immunogenic targets for T-cell recognition.

Actual binding of peptides to an HLA allele can be evaluated by stabilization of HLA expression on the antigen-processing defective cell line T2 (see, e.g., Xue et al., Prostate 30:73-8 (1997) and Peshwa et al., Prostate 36:129-38 (1998)). Immunogenicity of specific peptides can be evaluated *in vitro* by stimulation of CD8+ cytotoxic T lymphocytes (CTL) in the presence of antigen presenting cells such as dendritic cells.

It is to be appreciated that every epitope predicted by the BIMAS site, Epimer™ and Epimatrix™ sites, or specified by the HLA class I or class I motifs available in the art or which become part of the art such as set forth in Table IV (A) and Table IV (B) are to be "applied" to the 55P4H4 protein. As used in this context "applied" means that the 55P4H4 protein is evaluated, e.g., visually or by computer-based patterns finding methods, as appreciated by those of skill in the relevant art. Every subsequence of the 55P4H4 of 8, 9, 10, or 11 amino acid residues that bears an HLA Class I motif, or a subsequence of 9 or more amino acid residues that bear an HLA Class II motif are within the scope of the invention.

#### IV.B.) Expression of 55P4H4-related Proteins

In an embodiment described in the examples that follow, 55P4H4 can be conveniently expressed in cells (such as 293T cells) transfected with a commercially available expression vector such as a CMV-driven expression vector encoding 55P4H4 with a C-terminal 6XHis and MYC tag (pcDNA3.1/mycHIS, Invitrogen or Tag5, GenHunter Corporation, Nashville TN). The Tag5 vector provides an IgGK secretion signal that can be used to facilitate the production of a secreted 55P4H4 protein in transfected cells. The secreted HIS-tagged 55P4H4 in the culture media can be purified, e.g., using a nickel column using standard techniques.

#### IV.C.) Modifications of 55P4H4-related Proteins

Modifications of 55P4H4-related proteins such as covalent modifications are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a 55P4H4 polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the 55P4H4. Another type of covalent modification of the 55P4H4 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of a protein of the invention. Another type of covalent modification of 55P4H4 comprises linking the 55P4H4 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The 55P4H4-related proteins of the present invention can also be modified to form a chimeric molecule comprising 55P4H4 fused to another, heterologous polypeptide or amino acid sequence. Such a chimeric molecule can be synthesized chemically or recombinantly. A chimeric molecule can have a protein of the invention fused to another tumor-associated antigen or fragment thereof. Alternatively, a protein in accordance with the invention can comprise a fusion of fragments of the 55P4H4 sequence (amino or nucleic acid) such that a molecule is created that is not, through its length, directly homologous to the amino or nucleic acid sequences respectively of Figure 2. Such a chimeric molecule can comprise multiples of the same subsequence of 55P4H4. A chimeric molecule can comprise a fusion of a 55P4H4-related protein with a polyhistidine epitope tag, which provides an epitope to which immobilized nickel can selectively bind, with cytokines or with growth factors. The epitope tag is generally placed at the amino- or carboxyl- terminus of the 55P4H4. In an alternative embodiment, the chimeric molecule can comprise a fusion of a 55P4H4-related protein with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule (also referred to as an "immunoadhesin"), such a fusion could be to the Fc region of an IgG molecule. The Ig fusions preferably include the substitution of a soluble (transmembrane domain deleted or inactivated) form of a 55P4H4 polypeptide in place of at least one variable region within an Ig molecule. In a preferred embodiment, the immunoglobulin fusion includes the hinge, CH2 and CH3, or the hinge, CH1, CH2 and CH3 regions of an IgG1 molecule. For the production of immunoglobulin fusions see, e.g., U.S. Patent No. 5,428,130 issued June 27, 1995.

#### **IV.D.) Uses of 55P4H4-related Proteins**

The proteins of the invention have a number of different specific uses. As 55P4H4 is highly expressed in prostate and other cancers, 55P4H4-related proteins are used in methods that assess the status of 55P4H4 gene products in normal versus cancerous tissues, thereby elucidating the malignant phenotype. Typically, polypeptides from specific regions of the 55P4H4 protein are used to assess the presence of perturbations (such as deletions, insertions, point mutations etc.) in those regions (such as regions containing one or more motifs). Exemplary assays utilize antibodies or T cells targeting 55P4H4-related proteins comprising the amino acid residues of one or more of the biological motifs contained within the 55P4H4 polypeptide sequence in order to evaluate the characteristics of this region in normal versus cancerous tissues or to elicit an immune response to the epitope. Alternatively, 55P4H4-related proteins that contain the amino acid residues of one or more of the biological motifs in the 55P4H4 protein are used to screen for factors that interact with that region of 55P4H4.

55P4H4 protein fragments/subsequences are particularly useful in generating and characterizing domain-specific antibodies (e.g., antibodies recognizing an extracellular or intracellular epitope of an 55P4H4 protein), for identifying agents or cellular factors that bind to 55P4H4 or a particular structural domain thereof, and in various therapeutic and diagnostic contexts, including but not limited to diagnostic assays, cancer vaccines and methods of preparing such vaccines.



Proteins encoded by the 55P4H4 genes, or by analogs, homologs or fragments thereof, have a variety of uses, including but not limited to generating antibodies and in methods for identifying ligands and other agents and cellular constituents that bind to an 55P4H4 gene product. Antibodies raised against an 55P4H4 protein or fragment thereof are useful in diagnostic and prognostic assays, and imaging methodologies in the management of human cancers characterized by expression of 55P4H4 protein, such as those listed in Table I. Such antibodies can be expressed intracellularly and used in methods of treating patients with such cancers. 55P4H4-related nucleic acids or proteins are also used in generating HTL or CTL responses.

Various immunological assays useful for the detection of 55P4H4 proteins are used, including but not limited to various types of radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), enzyme-linked immunofluorescent assays (ELIFA), immunocytochemical methods, and the like. Antibodies can be labeled and used as immunological imaging reagents capable of detecting 55P4H4-expressing cells (e.g., in radioscintigraphic imaging methods). 55P4H4-proteins are also particularly useful in generating cancer vaccines, as further described herein.

#### V.) 55P4H4 Antibodies

Another aspect of the invention provides antibodies that bind to 55P4H4-related proteins. Preferred antibodies specifically bind to a 55P4H4-related protein and do not bind (or bind weakly) to peptides or proteins that are not 55P4H4-related proteins. For example, antibodies bind 55P4H4 can bind 55P4H4-related proteins such as the homologs or analogs thereof.

55P4H4 antibodies of the invention are particularly useful in prostate cancer diagnostic and prognostic assays, and imaging methodologies. Similarly, such antibodies are useful in the treatment, diagnosis, and/or prognosis of other cancers, to the extent 55P4H4 is also expressed or overexpressed in these other cancers. Moreover, intracellularly expressed antibodies (e.g., single chain antibodies) are therapeutically useful in treating cancers in which the expression of 55P4H4 is involved, such as advanced or metastatic prostate cancers.

The invention also provides various immunological assays useful for the detection and quantification of 55P4H4 and mutant 55P4H4-related proteins. Such assays can comprise one or more 55P4H4 antibodies capable of recognizing and binding a 55P4H4-related protein, as appropriate. These assays are performed within various immunological assay formats well known in the art, including but not limited to various types of radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), enzyme-linked immunofluorescent assays (ELIFA), and the like.

Immunological non-antibody assays of the invention also comprise T cell immunogenicity assays (inhibitory or stimulatory) as well as major histocompatibility complex (MHC) binding assays.

In addition, immunological imaging methods capable of detecting prostate cancer and other cancers expressing 55P4H4 are also provided by the invention, including but not limited to

radioscintigraphic imaging methods using labeled 55P4H4 antibodies. Such assays are clinically useful in the detection, monitoring, and prognosis of 55P4H4 expressing cancers such as prostate cancer.

55P4H4 antibodies are also used in methods for purifying a 55P4H4-related protein and for isolating 55P4H4 homologues and related molecules. For example, a method of purifying a 55P4H4-related protein comprises incubating an 55P4H4 antibody, which has been coupled to a solid matrix, with a lysate or other solution containing a 55P4H4-related protein under conditions that permit the 55P4H4 antibody to bind to the 55P4H4-related protein; washing the solid matrix to eliminate impurities; and eluting the 55P4H4-related protein from the coupled antibody. Other uses of the 55P4H4 antibodies of the invention include generating anti-idiotypic antibodies that mimic the 55P4H4 protein.

Various methods for the preparation of antibodies are well known in the art. For example, antibodies can be prepared by immunizing a suitable mammalian host using a 55P4H4-related protein, peptide, or fragment, in isolated or immunoconjugated form (Antibodies: A Laboratory Manual, CSH Press, Eds., Harlow, and Lane (1988); Harlow, Antibodies, Cold-Spring Harbor Press, NY (1989)). In addition, fusion proteins of 55P4H4 can also be used, such as a 55P4H4 GST-fusion protein. In a particular embodiment, a GST fusion protein comprising all or most of the amino acid sequence of Figure 2 or Figure 3 is produced, then used as an immunogen to generate appropriate antibodies. In another embodiment, a 55P4H4-related protein is synthesized and used as an immunogen.

In addition, naked DNA immunization techniques known in the art are used (with or without purified 55P4H4-related protein or 55P4H4 expressing cells) to generate an immune response to the encoded immunogen (for review, see Donnelly et al., 1997, Ann. Rev. Immunol. 15: 617-648).

The amino acid sequence of 55P4H4 as shown in Figure 2 or Figure 3 can be analyzed to select specific regions of the 55P4H4 protein for generating antibodies. For example, hydrophobicity and hydrophilicity analyses of the 55P4H4 amino acid sequence are used to identify hydrophilic regions in the 55P4H4 structure. Regions of the 55P4H4 protein that show immunogenic structure, as well as other regions and domains, can readily be identified using various other methods known in the art, such as Chou-Fasman, Garnier-Robson, Kyte-Doolittle, Eisenberg, Karplus-Schultz or Jameson-Wolf analysis. A number of these programs are available on the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) on the ExPasy molecular biology server, e.g., Hydrophilicity (Hopp T.P., Woods K.R., 1981. Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828), Hydropathicity, (Kyte J., Doolittle R.F., 1982. J. Mol. Biol. 157:105-132); Percentage Accessible Residues (Janin J., 1979 Nature 277:491-492) Average Flexibility, (Bhaskaran R., and Ponnuswamy P.K., 1988. Int. J. Pept. Protein Res. 32:242-255); Beta-turn (Deleage, G., Roux B. 1987 Protein Engineering 1:289-294); and others. Thus, each region identified by any of these programs or methods is within the scope of the present invention. Methods for the generation of 55P4H4 antibodies are further illustrated by way of the examples provided herein. Methods for preparing a protein or polypeptide for use as an immunogen are well known in the art. Also well known in the art are

methods for preparing immunogenic conjugates of a protein with a carrier, such as BSA, KLH or other carrier protein. In some circumstances, direct conjugation using, for example, carbodiimide reagents are used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, are effective. Administration of a 55P4H4 immunogen is often conducted by injection over a  
5 suitable time period and with use of a suitable adjuvant, as is understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

55P4H4 monoclonal antibodies can be produced by various means well known in the art. For example, immortalized cell lines that secrete a desired monoclonal antibody are prepared using the standard hybridoma technology of Kohler and Milstein or modifications that immortalize antibody-producing B  
10 cells, as is generally known. Immortalized cell lines that secrete the desired antibodies are screened by immunoassay in which the antigen is a 55P4H4-related protein. When the appropriate immortalized cell culture is identified, the cells can be expanded and antibodies produced either from *in vitro* cultures or from ascites fluid.

The antibodies or fragments of the invention can also be produced, by recombinant means.  
15 Regions that bind specifically to the desired regions of the 55P4H4 protein can also be produced in the context of chimeric or complementarity determining region (CDR) grafted antibodies of multiple species origin. Humanized or human 55P4H4 antibodies can also be produced, and are preferred for use in therapeutic contexts. Methods for humanizing murine and other non-human antibodies, by substituting one or more of the non-human antibody CDRs for corresponding human antibody sequences, are well known  
20 (see for example, Jones et al., 1986, Nature 321: 522-525; Riechmann et al., 1988, Nature 332: 323-327; Verhoeven et al., 1988, Science 239: 1534-1536). See also, Carter et al., 1993, Proc. Natl. Acad. Sci. USA 89: 4285 and Sims et al., 1993, J. Immunol. 151: 2296.

Methods for producing fully human monoclonal antibodies include phage display and transgenic methods (for review, see Vaughan et al., 1998, Nature Biotechnology 16: 535-539). Fully human 55P4H4  
25 monoclonal antibodies can be generated using cloning technologies employing large human Ig gene combinatorial libraries (i.e., phage display) (Griffiths and Hoogenboom, Building an *in vitro* immune system: human antibodies from phage display libraries. In: Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications in Man, Clark, M. (Ed.), Nottingham Academic, pp 45-64 (1993); Burton and Barbas, Human Antibodies from combinatorial libraries. *Id.*, pp 65-82). Fully human  
30 55P4H4 monoclonal antibodies can also be produced using transgenic mice engineered to contain human immunoglobulin gene loci as described in PCT Patent Application WO98/24893, Kucherlapati and Jakobovits et al., published December 3, 1997 (see also, Jakobovits, 1998, Exp. Opin. Invest. Drugs 7(4): 607-614; U.S. patents 6,162,963 issued 19 December 2000; 6,150,584 issued 12 November 2000; and, 6,114,598 issued 5 September 2000). This method avoids the *in vitro* manipulation required with phage  
35 display technology and efficiently produces high affinity authentic human antibodies.

Reactivity of 55P4H4 antibodies with an 55P4H4-related protein can be established by a number of well known means, including Western blot, immunoprecipitation, ELISA, and FACS analyses using, as appropriate, 55P4H4-related proteins, 55P4H4-expressing cells or extracts thereof. A 55P4H4 antibody or fragment thereof can be labeled with a detectable marker or conjugated to a second molecule. Suitable detectable markers include, but are not limited to, a radioisotope, a fluorescent compound, a bioluminescent compound, chemiluminescent compound, a metal chelator or an enzyme. Further, bi-specific antibodies specific for two or more 55P4H4 epitopes are generated using methods generally known in the art. Homodimeric antibodies can also be generated by cross-linking techniques known in the art (e.g., Wolff et al., Cancer Res. 53: 2560-2565).

10 **VI.) 55P4H4 Transgenic Animals**

Nucleic acids that encode a 55P4H4-related protein can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. In accordance with established techniques, cDNA encoding 55P4H4 can be used to clone genomic DNA that encodes 55P4H4. The cloned genomic sequences can then be used to generate transgenic animals containing cells that express DNA that encode 55P4H4. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 issued 12 April 1988, and 4,870,009 issued 26 September 1989. Typically, particular cells would be targeted for 55P4H4 transgene incorporation with tissue-specific enhancers.

Transgenic animals that include a copy of a transgene encoding 55P4H4 can be used to examine the effect of increased expression of DNA that encodes 55P4H4. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this aspect of the invention, an animal is treated with a reagent and a reduced incidence of a pathological condition, compared to untreated animals that bear the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, non-human homologues of 55P4H4 can be used to construct a 55P4H4 "knock out" animal that has a defective or altered gene encoding 55P4H4 as a result of homologous recombination between the endogenous gene encoding 55P4H4 and altered genomic DNA encoding 55P4H4 introduced into an embryonic cell of the animal. For example, cDNA that encodes 55P4H4 can be used to clone genomic DNA encoding 55P4H4 in accordance with established techniques. A portion of the genomic DNA encoding 55P4H4 can be deleted or replaced with another gene, such as a gene encoding a selectable marker that can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see, e.g., Li et al., Cell,

69:915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras (see, e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal, and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knock out animals can be characterized, for example, for their ability to defend against certain pathological conditions or for their development of pathological conditions due to absence of the 55P4H4 polypeptide.

10 **VII.) Methods for the Detection of 55P4H4**

Another aspect of the present invention relates to methods for detecting 55P4H4 polynucleotides and 55P4H4-related proteins, as well as methods for identifying a cell that expresses 55P4H4. The expression profile of 55P4H4 makes it a diagnostic marker for metastasized disease. Accordingly, the status of 55P4H4 gene products provides information useful for predicting a variety of factors including susceptibility to advanced stage disease, rate of progression, and/or tumor aggressiveness. As discussed in detail herein, the status of 55P4H4 gene products in patient samples can be analyzed by a variety of protocols that are well known in the art including immunohistochemical analysis, the variety of Northern blotting techniques including *in situ* hybridization, RT-PCR analysis (for example on laser capture micro-dissected samples), Western blot analysis and tissue array analysis.

More particularly, the invention provides assays for the detection of 55P4H4 polynucleotides in a biological sample, such as serum, bone, prostate, and other tissues, urine, semen, cell preparations, and the like. Detectable 55P4H4 polynucleotides include, for example, a 55P4H4 gene or fragment thereof, 55P4H4 mRNA, alternative splice variant 55P4H4 mRNAs, and recombinant DNA or RNA molecules that contain a 55P4H4 polynucleotide. A number of methods for amplifying and/or detecting the presence of 55P4H4 polynucleotides are well known in the art and can be employed in the practice of this aspect of the invention.

In one embodiment, a method for detecting an 55P4H4 mRNA in a biological sample comprises producing cDNA from the sample by reverse transcription using at least one primer; amplifying the cDNA so produced using an 55P4H4 polynucleotides as sense and antisense primers to amplify 55P4H4 cDNAs therein; and detecting the presence of the amplified 55P4H4 cDNA. Optionally, the sequence of the amplified 55P4H4 cDNA can be determined.

In another embodiment, a method of detecting a 55P4H4 gene in a biological sample comprises first isolating genomic DNA from the sample; amplifying the isolated genomic DNA using 55P4H4 polynucleotides as sense and antisense primers; and detecting the presence of the amplified 55P4H4 gene. Any number of appropriate sense and antisense probe combinations can be designed from the nucleotide sequence provided for the 55P4H4 (Figure 2) and used for this purpose.

The invention also provides assays for detecting the presence of an 55P4H4 protein in a tissue or other biological sample such as serum, semen, bone, prostate, urine, cell preparations, and the like. Methods for detecting a 55P4H4-related protein are also well known and include, for example, immunoprecipitation, immunohistochemical analysis, Western blot analysis, molecular binding assays, ELISA, ELIFA and the like. For example, a method of detecting the presence of a 55P4H4-related protein in a biological sample comprises first contacting the sample with a 55P4H4 antibody, a 55P4H4-reactive fragment thereof, or a recombinant protein containing an antigen binding region of a 55P4H4 antibody; and then detecting the binding of 55P4H4-related protein in the sample.

Methods for identifying a cell that expresses 55P4H4 are also within the scope of the invention. In one embodiment, an assay for identifying a cell that expresses a 55P4H4 gene comprises detecting the presence of 55P4H4 mRNA in the cell. Methods for the detection of particular mRNAs in cells are well known and include, for example, hybridization assays using complementary DNA probes (such as *in situ* hybridization using labeled 55P4H4 riboprobes, Northern-blot and related techniques) and various nucleic acid amplification assays (such as RT-PCR using complementary primers specific for 55P4H4, and other amplification type detection methods, such as, for example, branched DNA, SISBA, TMA and the like). Alternatively, an assay for identifying a cell that expresses a 55P4H4 gene comprises detecting the presence of 55P4H4-related protein in the cell or secreted by the cell. Various methods for the detection of proteins are well known in the art and are employed for the detection of 55P4H4-related proteins and cells that express 55P4H4-related proteins.

55P4H4 expression analysis is also useful as a tool for identifying and evaluating agents that modulate 55P4H4 gene expression. For example, 55P4H4 expression is significantly upregulated in prostate cancer, and is expressed in cancers of the tissues listed in Table I. Identification of a molecule or biological agent that inhibits 55P4H4 expression or over-expression in cancer cells is of therapeutic value. For example, such an agent can be identified by using a screen that quantifies 55P4H4 expression by RT-PCR, nucleic acid hybridization or antibody binding.

#### **VIII.) Methods for Monitoring the Status of 55P4H4-related Genes and Their Products**

Oncogenesis is known to be a multistep process where cellular growth becomes progressively dysregulated and cells progress from a normal physiological state to precancerous and then cancerous states (see, e.g., Alers et al., Lab Invest. 77(5): 437-438 (1997) and Isaacs et al., Cancer Surv. 23: 19-32 (1995)). In this context, examining a biological sample for evidence of dysregulated cell growth (such as aberrant 55P4H4 expression in cancers) allows for early detection of such aberrant physiology, before a pathologic state such as cancer has progressed to a stage that therapeutic options are more limited and or the prognosis is worse. In such examinations, the status of 55P4H4 in a biological sample of interest can be compared, for example, to the status of 55P4H4 in a corresponding normal sample (e.g. a sample from that individual or alternatively another individual that is not affected by a pathology). An alteration in the status of 55P4H4 in the biological sample (as compared to the normal

sample) provides evidence of dysregulated cellular growth. In addition to using a biological sample that is not affected by a pathology as a normal sample, one can also use a predetermined normative value such as a predetermined normal level of mRNA expression (see, e.g., Grever et al., J. Comp. Neurol. 1996 Dec 9;376(2):306-14 and U.S. Patent No. 5,837,501) to compare 55P4H4 status in a sample.

The term "status" in this context is used according to its art accepted meaning and refers to the condition or state of a gene and its products. Typically, skilled artisans use a number of parameters to evaluate the condition or state of a gene and its products. These include, but are not limited to the location of expressed gene products (including the location of 55P4H4 expressing cells) as well as the level, and biological activity of expressed gene products (such as 55P4H4 mRNA, polynucleotides and polypeptides). Typically, an alteration in the status of 55P4H4 comprises a change in the location of 55P4H4 and/or 55P4H4 expressing cells and/or an increase in 55P4H4 mRNA and/or protein expression.

55P4H4 status in a sample can be analyzed by a number of means well known in the art, including without limitation, immunohistochemical analysis, *in situ* hybridization, RT-PCR analysis on laser capture micro-dissected samples, Western blot analysis, and tissue array analysis. Typical protocols for evaluating the status of the 55P4H4 gene and gene products are found, for example in Ausubel et al. eds., 1995, Current Protocols In Molecular Biology, Units 2 (Northern Blotting), 4 (Southern Blotting), 15 (Immunoblotting) and 18 (PCR Analysis). Thus, the status of 55P4H4 in a biological sample is evaluated by various methods utilized by skilled artisans including, but not limited to genomic Southern analysis (to examine, for example perturbations in the 55P4H4 gene), Northern analysis and/or PCR analysis of 55P4H4 mRNA (to examine, for example alterations in the polynucleotide sequences or expression levels of 55P4H4 mRNAs), and, Western and/or immunohistochemical analysis (to examine, for example alterations in polypeptide sequences, alterations in polypeptide localization within a sample, alterations in expression levels of 55P4H4 proteins and/or associations of 55P4H4 proteins with polypeptide binding partners). Detectable 55P4H4 polynucleotides include, for example, a 55P4H4 gene or fragment thereof, 55P4H4 mRNA, alternative splice variants, 55P4H4 mRNAs, and recombinant DNA or RNA molecules containing a 55P4H4 polynucleotide.

The expression profile of 55P4H4 makes it a diagnostic marker for local and/or metastasized disease, and provides information on the growth or oncogenic potential of a biological sample. In particular, the status of 55P4H4 provides information useful for predicting susceptibility to particular disease stages, progression, and/or tumor aggressiveness. The invention provides methods and assays for determining 55P4H4 status and diagnosing cancers that express 55P4H4, such as cancers of the tissues listed in Table I. For example, because 55P4H4 mRNA is so highly expressed in prostate and other cancers relative to normal prostate tissue, assays that evaluate the levels of 55P4H4 mRNA transcripts or

proteins in a biological sample can be used to diagnose a disease associated with 55P4H4 dysregulation, and can provide prognostic information useful in defining appropriate therapeutic options.

The expression status of 55P4H4 provides information including the presence, stage and location of dysplastic, precancerous and cancerous cells, predicting susceptibility to various stages of disease, and/or for gauging tumor aggressiveness. Moreover, the expression profile makes it useful as an imaging reagent for metastasized disease. Consequently, an aspect of the invention is directed to the various molecular prognostic and diagnostic methods for examining the status of 55P4H4 in biological samples such as those from individuals suffering from, or suspected of suffering from a pathology characterized by dysregulated cellular growth, such as cancer.

As described above, the status of 55P4H4 in a biological sample can be examined by a number of well-known procedures in the art. For example, the status of 55P4H4 in a biological sample taken from a specific location in the body can be examined by evaluating the sample for the presence or absence of 55P4H4 expressing cells (e.g. those that express 55P4H4 mRNAs or proteins). This examination can provide evidence of dysregulated cellular growth, for example, when 55P4H4-expressing cells are found in a biological sample that does not normally contain such cells (such as a lymph node), because such alterations in the status of 55P4H4 in a biological sample are often associated with dysregulated cellular growth. Specifically, one indicator of dysregulated cellular growth is the metastases of cancer cells from an organ of origin (such as the prostate) to a different area of the body (such as a lymph node). In this context, evidence of dysregulated cellular growth is important for example because occult lymph node metastases can be detected in a substantial proportion of patients with prostate cancer, and such metastases are associated with known predictors of disease progression (see, e.g., Murphy et al., Prostate 42(4): 315-317 (2000); Su et al., Semin. Surg. Oncol. 18(1): 17-28 (2000) and Freeman et al., J Urol 1995 Aug 154(2 Pt 1):474-8).

In one aspect, the invention provides methods for monitoring 55P4H4 gene products by determining the status of 55P4H4 gene products expressed by cells from an individual suspected of having a disease associated with dysregulated cell growth (such as hyperplasia or cancer) and then comparing the status so determined to the status of 55P4H4 gene products in a corresponding normal sample. The presence of aberrant 55P4H4 gene products in the test sample relative to the normal sample provides an indication of the presence of dysregulated cell growth within the cells of the individual.

In another aspect, the invention provides assays useful in determining the presence of cancer in an individual, comprising detecting a significant increase in 55P4H4 mRNA or protein expression in a test cell or tissue sample relative to expression levels in the corresponding normal cell or tissue. The presence of 55P4H4 mRNA can, for example, be evaluated in tissue samples including but not limited to those listed in Table I. The presence of significant 55P4H4 expression in any of these tissues is



useful to indicate the emergence, presence and/or severity of a cancer, since the corresponding normal tissues do not express 55P4H4 mRNA or express it at lower levels.

5 In a related embodiment, 55P4H4 status is determined at the protein level rather than at the nucleic acid level. For example, such a method comprises determining the level of 55P4H4 protein expressed by cells in a test tissue sample and comparing the level so determined to the level of 55P4H4 expressed in a corresponding normal sample. In one embodiment, the presence of 55P4H4 protein is evaluated, for example, using immunohistochemical methods. 55P4H4 antibodies or binding partners capable of detecting 55P4H4 protein expression are used in a variety of assay formats well known in the art for this purpose.

10 In a further embodiment, one can evaluate the status 55P4H4 nucleotide and amino acid sequences in a biological sample in order to identify perturbations in the structure of these molecules. These perturbations can include insertions, deletions, substitutions and the like. Such evaluations are useful because perturbations in the nucleotide and amino acid sequences are observed in a large number of proteins associated with a growth dysregulated phenotype (see, e.g., Marrogi et al., 1999, J. Cutan. Pathol. 26(8):369-378). For example, a mutation in the sequence of 55P4H4 may be indicative of the presence or promotion of a tumor. Such assays therefore have diagnostic and predictive value where a mutation in 55P4H4 indicates a potential loss of function or increase in tumor growth.

15 A wide variety of assays for observing perturbations in nucleotide and amino acid sequences are well known in the art. For example, the size and structure of nucleic acid or amino acid sequences of 55P4H4 gene products are observed by the Northern, Southern, Western, PCR and DNA sequencing protocols discussed herein. In addition, other methods for observing perturbations in nucleotide and amino acid sequences such as single strand conformation polymorphism analysis are well known in the art (see, e.g., U.S. Patent Nos. 5,382,510 issued 7 September 1999, and 5,952,170 issued 17 January 1995).

20 Additionally, one can examine the methylation status of the 55P4H4 gene in a biological sample. Aberrant demethylation and/or hypermethylation of CpG islands in gene 5' regulatory regions frequently occurs in immortalized and transformed cells, and can result in altered expression of various genes. For example, promoter hypermethylation of the pi-class glutathione S-transferase (a protein expressed in normal prostate but not expressed in >90% of prostate carcinomas) appears to permanently silence transcription of this gene and is the most frequently detected genomic alteration in prostate carcinomas (De Marzo et al., Am. J. Pathol. 155(6): 1985-1992 (1999)). In addition, this alteration is present in at least 70% of cases of high-grade prostatic intraepithelial neoplasia (PIN) (Brooks et al, Cancer Epidemiol. Biomarkers Prev., 1998, 7:531-536). In another example, expression of the LAGE-I tumor specific gene (which is not expressed in normal prostate but is expressed in 25-50% of prostate cancers) is induced by deoxy-azacytidine in lymphoblastoid cells, suggesting that tumoral expression is due to demethylation (Lethe et al., Int. J. Cancer 76(6): 903-908 (1998)). A variety of assays for examining methylation status of a gene are well known in the art. For example, one can utilize, in Southern

hybridization approaches, methylation-sensitive restriction enzymes which cannot cleave sequences that contain methylated CpG sites to assess the methylation status of CpG islands. In addition, MSP (methylation specific PCR) can rapidly profile the methylation status of all the CpG sites present in a CpG island of a given gene. This procedure involves initial modification of DNA by sodium bisulfite (which will convert all unmethylated cytosines to uracil) followed by amplification using primers specific for methylated versus unmethylated DNA. Protocols involving methylation interference can also be found for example in Current Protocols In Molecular Biology, Unit 12, Frederick M. Ausubel et al. eds., 1995.

Gene amplification is an additional method for assessing the status of 5SP4H4. Gene amplification is measured in a sample directly, for example, by conventional Southern blotting or Northern blotting to quantitate the transcription of mRNA (Thomas, 1980, Proc. Natl. Acad. Sci. USA, 77:5201-5205), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies are employed that recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn are labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Biopsied tissue or peripheral blood can be conveniently assayed for the presence of cancer cells using for example, Northern, dot blot or RT-PCR analysis to detect 5SP4H4 expression. The presence of RT-PCR amplifiable 5SP4H4 mRNA provides an indication of the presence of cancer. RT-PCR assays are well known in the art. RT-PCR detection assays for tumor cells in peripheral blood are currently being evaluated for use in the diagnosis and management of a number of human solid tumors. In the prostate cancer field, these include RT-PCR assays for the detection of cells expressing PSA and PSM (Verkaik et al., 1997, Urol. Res. 25:373-384; Ghossein et al., 1995, J. Clin. Oncol. 13:1195-2000; Heston et al., 1995, Clin. Chem. 41:1687-1688).

A further aspect of the invention is an assessment of the susceptibility that an individual has for developing cancer. In one embodiment, a method for predicting susceptibility to cancer comprises detecting 5SP4H4 mRNA or 5SP4H4 protein in a tissue sample, its presence indicating susceptibility to cancer, wherein the degree of 5SP4H4 mRNA expression correlates to the degree of susceptibility. In a specific embodiment, the presence of 5SP4H4 in prostate or other tissue is examined, with the presence of 5SP4H4 in the sample providing an indication of prostate cancer susceptibility (or the emergence or existence of a prostate tumor). Similarly, one can evaluate the integrity 5SP4H4 nucleotide and amino acid sequences in a biological sample, in order to identify perturbations in the structure of these molecules such as insertions, deletions, substitutions and the like. The presence of one or more perturbations in 5SP4H4 gene products in the sample is an indication of cancer susceptibility (or the emergence or existence of a tumor).

The invention also comprises methods for gauging tumor aggressiveness. In one embodiment, a method for gauging aggressiveness of a tumor comprises determining the level of 55P4H4 mRNA or 55P4H4 protein expressed by tumor cells, comparing the level so determined to the level of 55P4H4 mRNA or 55P4H4 protein expressed in a corresponding normal tissue taken from the same individual or a normal tissue reference sample, wherein the degree of 55P4H4 mRNA or 55P4H4 protein expression in the tumor sample relative to the normal sample indicates the degree of aggressiveness. In a specific embodiment, aggressiveness of a tumor is evaluated by determining the extent to which 55P4H4 is expressed in the tumor cells, with higher expression levels indicating more aggressive tumors. Another embodiment is the evaluation of the integrity of 55P4H4 nucleotide and amino acid sequences in a biological sample, in order to identify perturbations in the structure of these molecules such as insertions, deletions, substitutions and the like. The presence of one or more perturbations indicates more aggressive tumors.

Another embodiment of the invention is directed to methods for observing the progression of a malignancy in an individual over time. In one embodiment, methods for observing the progression of a malignancy in an individual over time comprise determining the level of 55P4H4 mRNA or 55P4H4 protein expressed by cells in a sample of the tumor, comparing the level so determined to the level of 55P4H4 mRNA or 55P4H4 protein expressed in an equivalent tissue sample taken from the same individual at a different time, wherein the degree of 55P4H4 mRNA or 55P4H4 protein expression in the tumor sample over time provides information on the progression of the cancer. In a specific embodiment, the progression of a cancer is evaluated by determining 55P4H4 expression in the tumor cells over time, where increased expression over time indicates a progression of the cancer. Also, one can evaluate the integrity 55P4H4 nucleotide and amino acid sequences in a biological sample in order to identify perturbations in the structure of these molecules such as insertions, deletions, substitutions and the like, where the presence of one or more perturbations indicates a progression of the cancer.

The above diagnostic approaches can be combined with any one of a wide variety of prognostic and diagnostic protocols known in the art. For example, another embodiment of the invention is directed to methods for observing a coincidence between the expression of 55P4H4 gene and 55P4H4 gene products (or perturbations in 55P4H4 gene and 55P4H4 gene products) and a factor that is associated with malignancy, as a means for diagnosing and prognosticating the status of a tissue sample. A wide variety of factors associated with malignancy can be utilized, such as the expression of genes associated with malignancy (e.g. PSA, PSCA and PSM expression for prostate cancer etc.) as well as gross cytological observations (see, e.g., Bocking et al., 1984, Anal. Quant. Cytol. 6(2):74-88; Epstein, 1995, Hum. Pathol. 26(2):223-9; Thorson et al., 1998, Mod. Pathol. 11(6):543-51; Baisden et al., 1999, Am. J. Surg. Pathol. 23(8):918-24). Methods for observing a coincidence between the expression of 55P4H4 gene and 55P4H4 gene products (or perturbations in 55P4H4 gene and 55P4H4 gene products) and another factor that is associated with malignancy are useful, for example, because the presence of a set of specific factors

that coincide with disease provides information crucial for diagnosing and prognosticating the status of a tissue sample.

5 In one embodiment, methods for observing a coincidence between the expression of 55P4H4 gene and 55P4H4 gene products (or perturbations in 55P4H4 gene and 55P4H4 gene products) and another factor associated with malignancy entails detecting the overexpression of 55P4H4 mRNA or protein in a tissue sample, detecting the overexpression of PSA mRNA or protein in a tissue sample (or PSCA or PSM expression), and observing a coincidence of 55P4H4 mRNA or protein and PSA mRNA or protein overexpression (or PSCA or PSM expression). In a specific embodiment, the expression of 55P4H4 and PSA mRNA in prostate tissue is examined, where the coincidence of 55P4H4 and PSA mRNA overexpression in the sample indicates the existence of prostate cancer, prostate cancer susceptibility or the emergence or status of a prostate tumor.

10 Methods for detecting and quantifying the expression of 55P4H4 mRNA or protein are described herein, and standard nucleic acid and protein detection and quantification technologies are well-known in the art. Standard methods for the detection and quantification of 55P4H4 mRNA include *in situ* hybridization using labeled 55P4H4 riboprobes, Northern blot and related techniques using 55P4H4 polynucleotide probes, RT-PCR analysis using primers specific for 55P4H4, and other amplification type detection methods, such as, for example, branched DNA, SISBA, TMA and the like. In a specific embodiment, semi-quantitative RT-PCR is used to detect and quantify 55P4H4 mRNA expression. Any number of primers capable of amplifying 55P4H4 can be used for this purpose, including but not limited to the various primer sets specifically described herein. In a specific embodiment, polyclonal or monoclonal antibodies specifically reactive with the wild-type 55P4H4 protein can be used in an immunohistochemical assay of biopsied tissue.

#### **IX.) Identification of Molecules That Interact With 55P4H4**

25 The 55P4H4 protein and nucleic acid sequences disclosed herein allow a skilled artisan to identify proteins, small molecules and other agents that interact with 55P4H4, as well as pathways activated by 55P4H4 via any one of a variety of art accepted protocols. For example, one can utilize one of the so-called interaction trap systems (also referred to as the "two-hybrid assay"). In such systems, molecules interact and reconstitute a transcription factor which directs expression of a reporter gene, whereupon the expression of the reporter gene is assayed. Other systems identify protein-protein interactions *in vivo* through reconstitution of a eukaryotic transcriptional activator, see, e.g., U.S. Patent Nos. 5,955,280 issued 21 September 1999, 5,925,523 issued 20 July 1999, 5,846,722 issued 8 December 1998 and 6,004,746 issued 21 December 1999. Algorithms are also available in the art for genome-based predictions of protein function (see, e.g., Marcotte, et al., Nature 402: 4 November 1999, 83-86).

35 Alternatively one can screen peptide libraries to identify molecules that interact with 55P4H4 protein sequences. In such methods, peptides that bind to a molecule such as 55P4H4 are identified by

screening libraries that encode a random or controlled collection of amino acids. Peptides encoded by the libraries are expressed as fusion proteins of bacteriophage coat proteins, the bacteriophage particles are then screened against the protein of interest.

5 Accordingly, peptides having a wide variety of uses, such as therapeutic, prognostic or diagnostic reagents, are thus identified without any prior information on the structure of the expected ligand or receptor molecule. Typical peptide libraries and screening methods that can be used to identify molecules that interact with 55P4H4 protein sequences are disclosed for example in U.S. Patent Nos. 5,723,286 issued 3 March 1998 and 5,733,731 issued 31 March 1998.

10 Alternatively, cell lines that express 55P4H4 are used to identify protein-protein interactions mediated by 55P4H4. Such interactions can be examined using immunoprecipitation techniques (see, e.g., Hamilton BJ, et al. Biochem. Biophys. Res. Commun. 1999, 261:646-51). 55P4H4 protein can be immunoprecipitated from 55P4H4-expressing cell lines using anti-55P4H4 antibodies. Alternatively, antibodies against His-tag can be used in a cell-line engineered to express 55P4H4 (vectors mentioned above). The immunoprecipitated complex can be examined for protein association by procedures such as Western blotting, <sup>35</sup>S-methionine labeling of proteins, protein microsequencing, silver staining and two-dimensional gel electrophoresis.

15 Small molecules and ligands that interact with 55P4H4 can be identified through related embodiments of such screening assays. For example, small molecules can be identified that interfere with protein function, including molecules that interfere with 55P4H4's ability to mediate phosphorylation and de-phosphorylation, interaction with DNA or RNA molecules as an indication of regulation of cell cycles, second messenger signaling or tumorigenesis. Similarly, small molecules that modulate ion channel, protein pump, or cell communication function of 55P4H4 are identified and used to treat patients that have a cancer that expresses the 55P4H4 antigen (see, e.g., Hille, B., Ionic Channels of Excitable Membranes 2<sup>nd</sup> Ed., Sinauer Assoc., Sunderland, MA, 1992). Moreover, ligands that regulate 55P4H4 function can be identified based on their ability to bind 55P4H4 and activate a reporter construct. Typical methods are discussed for example in U.S. Patent No. 5,928,868 issued 27 July 1999, and include methods for forming hybrid ligands in which at least one ligand is a small molecule. In an illustrative embodiment, cells engineered to express a fusion protein of 55P4H4 and a DNA-binding protein are used to co-express a fusion protein of a hybrid ligand/small molecule and a cDNA library transcriptional activator protein. The cells further contain a reporter gene, the expression of which is conditioned on the proximity of the first and second fusion proteins to each other, an event that occurs only if the hybrid ligand binds to target sites on both hybrid proteins. Those cells that express the reporter gene are selected and the unknown small molecule or the unknown ligand is identified. This method provides a means of identifying both activators and inhibitors of 55P4H4.

35 An embodiment of this invention comprises a method of screening for a molecule that interacts with an 55P4H4 amino acid sequence shown in Figure 2 and Figure 3 (SEQ ID NO: 2),

comprising the steps of contacting a population of molecules with the 55P4H4 amino acid sequence, allowing the population of molecules and the 55P4H4 amino acid sequence to interact under conditions that facilitate an interaction, determining the presence of a molecule that interacts with the 55P4H4 amino acid sequence, and then separating molecules that do not interact with the 55P4H4 amino acid sequence from molecules that do. In a specific embodiment, the method further comprises purifying a molecule that interacts with the 55P4H4 amino acid sequence. The identified molecule can be used to modulate a function performed by 55P4H4. In a preferred embodiment, the 55P4H4 amino acid sequence is contacted with a library of peptides.

#### **X.) Therapeutic Methods and Compositions**

10 The identification of 55P4H4 as a protein that is normally expressed in a restricted set of tissues, but which is also expressed in prostate and other cancers, opens a number of therapeutic approaches to the treatment of such cancers. As discussed herein, it is possible that 55P4H4 functions as a transcription factor involved in activating tumor-promoting genes or repressing genes that block tumorigenesis.

15 Accordingly, therapeutic approaches that inhibit the activity of the 55P4H4 protein are useful for patients suffering from a cancer that expresses 55P4H4. These therapeutic approaches generally fall into two classes. One class comprises various methods for inhibiting the binding or association of the 55P4H4 protein with its binding partner or with other proteins. Another class comprises a variety of methods for inhibiting the transcription of the 55P4H4 gene or translation of 55P4H4 mRNA.

#### **20 X.A.) 55P4H4 as a Target for Antibody-Based Therapy**

55P4H4 is an attractive target for antibody-based therapeutic strategies. A number of antibody strategies are known in the art for targeting both extracellular and intracellular molecules (see, e.g., complement and ADCC mediated killing as well as the use of intrabodies). Because 55P4H4 is expressed by cancer cells of various lineages and not by corresponding normal cells, systemic administration of 55P4H4-immunoreactive compositions are prepared that exhibit excellent sensitivity without toxic, non-specific and/or non-target effects caused by binding of the immunoreactive composition to non-target organs and tissues. Antibodies specifically reactive with domains of 55P4H4 are useful to treat 55P4H4-expressing cancers systemically, either as conjugates with a toxin or therapeutic agent, or as naked antibodies capable of inhibiting cell proliferation or function.

30 55P4H4 antibodies can be introduced into a patient such that the antibody binds to 55P4H4 and modulates a function, such as an interaction with a binding partner, and consequently mediates destruction of the tumor cells and/or inhibits the growth of the tumor cells. Mechanisms by which such antibodies exert a therapeutic effect can include complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, modulation of the physiological function of 55P4H4, inhibition of ligand binding or signal transduction pathways, modulation of tumor cell differentiation, alteration of tumor angiogenesis factor profiles, and/or apoptosis.

Those skilled in the art understand that antibodies can be used to specifically target and bind immunogenic molecules such as an immunogenic region of the 55P4H4 sequence shown in Figure 2. In addition, skilled artisans understand that it is routine to conjugate antibodies to cytotoxic agents. When cytotoxic and/or therapeutic agents are delivered directly to cells, such as by conjugating them to antibodies specific for a molecule expressed by that cell (e.g. 55P4H4), the cytotoxic agent will exert its known biological effect (i.e. cytotoxicity) on those cells.

A wide variety of compositions and methods for using antibody-cytotoxic agent conjugates to kill cells are known in the art. In the context of cancers, typical methods entail administering to an animal having a tumor a biologically effective amount of a conjugate comprising a selected cytotoxic and/or therapeutic agent linked to a targeting agent (e.g. an anti-55P4H4 antibody) that binds to a marker (e.g. 55P4H4) expressed, accessible to binding or localized on the cell surfaces. A typical embodiment is a method of delivering a cytotoxic and/or therapeutic agent to a cell expressing 55P4H4, comprising conjugating the cytotoxic agent to an antibody that immunospecifically binds to a 55P4H4 epitope, and, exposing the cell to the antibody-agent conjugate. Another illustrative embodiment is a method of treating an individual suspected of suffering from metastasized cancer, comprising a step of administering parenterally to said individual a pharmaceutical composition comprising a therapeutically effective amount of an antibody conjugated to a cytotoxic and/or therapeutic agent.

Cancer immunotherapy using anti-55P4H4 antibodies can be done in accordance with various approaches that have been successfully employed in the treatment of other types of cancer, including but not limited to colon cancer (Arlen et al., 1998, Crit. Rev. Immunol. 18:133-138), multiple myeloma (Ozaki et al., 1997, Blood 90:3179-3186, Tsunenari et al., 1997, Blood 90:2437-2444), gastric cancer (Kasprzyk et al., 1992, Cancer Res. 52:2771-2776), B-cell lymphoma (Funakoshi et al., 1996, J. Immunother. Emphasis Tumor Immunol. 19:93-101), leukemia (Zhong et al., 1996, Leuk. Res. 20:581-589), colorectal cancer (Moun et al., 1994, Cancer Res. 54:6160-6166; Velders et al., 1995, Cancer Res. 55:4398-4403), and breast cancer (Shepard et al., 1991, J. Clin. Immunol. 11:117-127). Some therapeutic approaches involve conjugation of naked antibody to a toxin, such as the conjugation of Y<sup>91</sup> or I<sup>131</sup> to anti-CD20 antibodies (e.g., Zevalin<sup>TM</sup>, IDEC Pharmaceuticals Corp. or Bexxar<sup>TM</sup>, Coulter Pharmaceuticals), while others involve co-administration of antibodies and other therapeutic agents, such as Herceptin<sup>TM</sup> (trastuzumab) with paclitaxel (Genentech, Inc.). To treat prostate cancer, for example, 55P4H4 antibodies can be administered in conjunction with radiation, chemotherapy or hormone ablation.

Although 55P4H4 antibody therapy is useful for all stages of cancer, antibody therapy can be particularly appropriate in advanced or metastatic cancers. Treatment with the antibody therapy of the invention is indicated for patients who have received one or more rounds of chemotherapy. Alternatively, antibody therapy of the invention is combined with a chemotherapeutic or radiation regimen for patients who have not received chemotherapeutic treatment. Additionally, antibody

therapy can enable the use of reduced dosages of concomitant chemotherapy, particularly for patients who do not tolerate the toxicity of the chemotherapeutic agent very well.

5 Cancer patients can be evaluated for the presence and level of 5SP4H4 expression, preferably using immunohistochemical assessments of tumor tissue, quantitative 5SP4H4 imaging, or other techniques that reliably indicate the presence and degree of 5SP4H4 expression. Immunohistochemical analysis of tumor biopsies or surgical specimens is preferred for this purpose. Methods for immunohistochemical analysis of tumor tissues are well known in the art.

10 Anti-5SP4H4 monoclonal antibodies that treat prostate and other cancers include those that initiate a potent immune response against the tumor or those that are directly cytotoxic. In this regard, anti-5SP4H4 monoclonal antibodies (mAbs) can elicit tumor cell lysis by either complement-mediated or antibody-dependent cell cytotoxicity (ADCC) mechanisms, both of which require an intact Fc portion of the immunoglobulin molecule for interaction with effector cell Fc receptor sites on complement proteins. In addition, anti-5SP4H4 mAbs that exert a direct biological effect on tumor growth are useful to treat cancers that express 5SP4H4. Mechanisms by which directly cytotoxic mAbs  
15 act include: inhibition of cell growth, modulation of cellular differentiation, modulation of tumor angiogenesis factor profiles, and the induction of apoptosis. The mechanism(s) by which a particular anti-5SP4H4 mAb exerts an anti-tumor effect is evaluated using any number of *in vitro* assays that evaluate cell death such as ADCC, ADMMC, complement-mediated cell lysis, and so forth, as is generally known in the art.

20 In some patients, the use of murine or other non-human monoclonal antibodies, or human/mouse chimeric mAbs can induce moderate to strong immune responses against the non-human antibody. This can result in clearance of the antibody from circulation and reduced efficacy. In the most severe cases, such an immune response can lead to the extensive formation of immune complexes which, potentially, can cause renal failure. Accordingly, preferred monoclonal antibodies used in the  
25 therapeutic methods of the invention are those that are either fully human or humanized and that bind specifically to the target 5SP4H4 antigen with high affinity but exhibit low or no antigenicity in the patient.

Therapeutic methods of the invention contemplate the administration of single anti-5SP4H4 mAbs as well as combinations, or cocktails, of different mAbs. Such mAb cocktails can have certain  
30 advantages inasmuch as they contain mAbs that target different epitopes, exploit different effector mechanisms or combine directly cytotoxic mAbs with mAbs that rely on immune effector functionality. Such mAbs in combination can exhibit synergistic therapeutic effects. In addition, anti-5SP4H4 mAbs can be administered concomitantly with other therapeutic modalities, including but not limited to various chemotherapeutic agents, androgen-blockers, immune modulators (e.g., IL-2, GM-CSF), surgery or radiation. The anti-5SP4H4 mAbs are administered in their "naked" or unconjugated  
35 form, or can have a therapeutic agent(s) conjugated to them.



Anti-55P4H4 antibody formulations are administered via any route capable of delivering the antibodies to a tumor cell. Routes of administration include, but are not limited to, intravenous, intraperitoneal, intramuscular, intratumor, intradermal, and the like. Treatment generally involves repeated administration of the anti-55P4H4 antibody preparation, via an acceptable route of administration such as intravenous injection (IV), typically at a dose in the range of about 0.1 to about 10 mg/kg body weight. In general, doses in the range of 10-500 mg mAb per week are effective and well tolerated.

Based on clinical experience with the Herceptin mAb in the treatment of metastatic breast cancer, an initial loading dose of approximately 4 mg/kg patient body weight IV, followed by weekly doses of about 2 mg/kg IV of the anti- 55P4H4 mAb preparation represents an acceptable dosing regimen. Preferably, the initial loading dose is administered as a 90 minute or longer infusion. The periodic maintenance dose is administered as a 30 minute or longer infusion, provided the initial dose was well tolerated. As appreciated by those of skill in the art, various factors can influence the ideal dose regimen in a particular case. Such factors include, for example, the binding affinity and half life of the Ab or mAbs used, the degree of 55P4H4 expression in the patient, the extent of circulating shed 55P4H4 antigen, the desired steady-state antibody concentration level, frequency of treatment, and the influence of chemotherapeutic or other agents used in combination with the treatment method of the invention, as well as the health status of a particular patient.

Optionally, patients should be evaluated for the levels of 55P4H4 in a given sample (e.g. the levels of circulating 55P4H4 antigen and/or 55P4H4 expressing cells) in order to assist in the determination of the most effective dosing regimen, etc. Such evaluations are also used for monitoring purposes throughout therapy, and are useful to gauge therapeutic success in combination with the evaluation of other parameters (such as serum PSA levels in prostate cancer therapy).

#### X.B.) Anti-Cancer Vaccines

The invention further provides cancer vaccines comprising a 55P4H4-related protein or 55P4H4-related nucleic acid. In view of the expression of 55P4H4, cancer vaccines prevent and/or treat 55P4H4-expressing cancers without creating non-specific effects on non-target tissues. The use of a tumor antigen in a vaccine that generates humoral and/or cell-mediated immune responses as anti-cancer therapy is well known in the art and has been employed in prostate cancer using human PSMA and rodent PAP immunogens (Hodge et al., 1995, Int. J. Cancer 63:231-237; Fong et al., 1997, J. Immunol. 159:3113-3117).

Genetic immunization methods can be employed to generate prophylactic or therapeutic humoral and cellular immune responses directed against cancer cells expressing 55P4H4. Constructs comprising DNA encoding a 55P4H4-related protein/immunogen and appropriate regulatory sequences can be injected directly into muscle or skin of an individual, such that the cells of the muscle or skin take-up the construct and express the encoded 55P4H4 protein/immunogen. Alternatively, a vaccine

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comprises a 55P4H4-related protein. Expression of the 55P4H4-related protein immunogen results in the generation of prophylactic or therapeutic humoral and cellular immunity against cells that bear 55P4H4 protein. Various prophylactic and therapeutic genetic immunization techniques known in the art can be used (for review, see information and references published at Internet address [www.genweb.com](http://www.genweb.com)).

5 Such methods can be readily practiced by employing a 55P4H4-related protein, or an 55P4H4-encoding nucleic acid molecule and recombinant vectors capable of expressing and presenting the 55P4H4 immunogen (which typically comprises a number of antibody or T cell epitopes). Skilled artisans understand that a wide variety of vaccine systems for delivery of immunoreactive epitopes are known in the art (see, e.g., Heryln et al., Ann Med 1999 Feb 31(1):66-78; Maruyama et al., Cancer Immunol Immunother 2000 Jun 49(3):123-32). Briefly, such methods of generating an immune response (e.g. humoral and/or cell-mediated) in a mammal, comprise the steps of: exposing the mammal's immune system to an immunoreactive epitope (e.g. an epitope present in the 55P4H4 protein shown in SEQ ID NO: 2 or analog or homolog thereof) so that the mammal generates an immune response that is specific for that epitope (e.g. generates antibodies that specifically recognize that epitope). In a preferred method, the 55P4H4 immunogen contains a biological motif.

CTL epitopes can be determined using specific algorithms to identify peptides within 55P4H4 protein that are capable of optimally binding to specified HLA alleles (e.g., Table IV (A) and Table IV (B); Epimer™ and Epimatrix™, Brown University ([http://www.brown.edu/Research/TB-HIV\\_Lab/epimatrix/epimatrix.html](http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html)); and, BIMAS, (<http://bimas.dcrt.nih.gov/>)). In a preferred embodiment, the 55P4H4 immunogen contains one or more amino acid sequences identified using one of the pertinent analytical techniques well known in the art, such as the sequences shown in Tables V-XVIII or a peptide of 8, 9, 10 or 11 amino acids specified by an HLA Class I motif (e.g., Table IV (A)) and/or a peptide of at least 9 amino acids that comprises an HLA Class II motif (e.g., Table IV (B)).

25 As is appreciated in the art, the HLA Class I binding groove is essentially closed ended so that peptides of only a particular size range can fit into the groove and be bound, generally HLA Class I epitopes are 8, 9, 10, or 11 amino acids long. In contrast, the HLA Class II binding groove is essentially open ended; therefore a peptide of about 9 or more amino acids can be bound by an HLA Class II molecule. Due to the binding groove differences between HLA Class I and II, HLA Class I motifs are length specific, i.e., position two of a Class I motif is the second amino acid in an amino to carboxyl direction of the peptide. The amino acid positions in a Class II motif are relative only to each other, not the overall peptide, i.e., additional amino acids can be attached to the amino and/or carboxyl termini of a motif-bearing sequence. HLA Class II epitopes are often 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids long, or longer than 25 amino acids.

35 A wide variety of methods for generating an immune response in a mammal are known in the art (for example as the first step in the generation of hybridomas). Methods of generating an immune

response in a mammal comprise exposing the mammal's immune system to an immunogenic epitope on a protein (e.g. the 55P4H4 protein) so that an immune response is generated. A typical embodiment consists of a method for generating an immune response to 55P4H4 in a host, by contacting the host with a sufficient amount of at least one 55P4H4 B cell or cytotoxic T-cell epitope or analog thereof; and at least one periodic interval thereafter re-contacting the host with the 55P4H4 B cell or cytotoxic T-cell epitope or analog thereof. A specific embodiment consists of a method of generating an immune response against a 55P4H4-related protein or a man-made multiepitopic peptide comprising: administering 55P4H4 immunogen (e.g. the 55P4H4 protein or a peptide fragment thereof, an 55P4H4 fusion protein or analog etc.) in a vaccine preparation to a human or another mammal. Typically, such vaccine preparations further contain a suitable adjuvant (see, e.g., U.S. Patent No. 6,146,635) or a universal helper epitope such as a PADRE<sup>TM</sup> peptide (Epimmune Inc., San Diego, CA; see, e.g., Alexander et al., J. Immunol. 2000 164(3); 164(3): 1625-1633; Alexander et al., Immunity 1994 1(9): 751-761 and Alexander et al., Immunol. Res. 1998 18(2): 79-92). An alternative method comprises generating an immune response in an individual against a 55P4H4 immunogen by: administering *in vivo* to muscle or skin of the individual's body a DNA molecule that comprises a DNA sequence that encodes an 55P4H4 immunogen, the DNA sequence operatively linked to regulatory sequences which control the expression of the DNA sequence; wherein the DNA molecule is taken up by cells, the DNA sequence is expressed in the cells and an immune response is generated against the immunogen (see, e.g., U.S. Patent No. 5,962,428). The DNA can be dissociated from an infectious agent. Optionally a genetic vaccine facilitator such as anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea is also administered.

Thus, viral gene delivery systems are used to deliver a 55P4H4-related nucleic acid molecule. Various viral gene delivery systems that can be used in the practice of the invention include, but are not limited to, vaccinia, fowlpox, canarypox, adenovirus, influenza, poliovirus, adeno-associated virus, lentivirus, and sindbis virus (Restifo, 1996, Curr. Opin. Immunol. 8:658-663). Non-viral delivery systems can also be employed by introducing naked DNA encoding a 55P4H4-related protein into the patient (e.g., intramuscularly or intradermally) to induce an anti-tumor response. In one embodiment, the full-length human 55P4H4 cDNA is employed. In another embodiment, 55P4H4 nucleic acid molecules encoding specific cytotoxic T lymphocyte (CTL) and/or antibody epitopes are employed.

Various *ex vivo* strategies can also be employed to generate an immune response. One approach involves the use of antigen presenting cells (APCs) such as dendritic cells to present 55P4H4 antigen to a patient's immune system. Dendritic cells express MHC class I and II molecules, B7 co-stimulator, and IL-12, and are thus highly specialized antigen presenting cells. In prostate cancer, autologous dendritic cells pulsed with peptides of the prostate-specific membrane antigen (PSMA) are being used in a Phase I clinical trial to stimulate prostate cancer patients' immune systems (Tjoa et al., 1996, Prostate 28:65-69; Murphy et al., 1996, Prostate 29:371-380). Thus, dendritic cells can be used to present 55P4H4

peptides to T cells in the context of MHC class I or II molecules. In one embodiment, autologous dendritic cells are pulsed with 55P4H4 peptides capable of binding to MHC class I and/or class II molecules. In another embodiment, dendritic cells are pulsed with the complete 55P4H4 protein. Yet another embodiment involves engineering the overexpression of the 55P4H4 gene in dendritic cells using various implementing vectors known in the art, such as adenovirus (Arthur et al., 1997, Cancer Gene Ther. 4:17-25), retrovirus (Henderson et al., 1996, Cancer Res. 56:3763-3770), lentivirus, adeno-associated virus, DNA transfection (Ribas et al., 1997, Cancer Res. 57:2865-2869), or tumor-derived RNA transfection (Ashley et al., 1997, J. Exp. Med. 186:1177-1182). Cells that express 55P4H4 can also be engineered to express immune modulators, such as GM-CSF, and used as immunizing agents.

Anti-idiotypic anti-55P4H4 antibodies can also be used in anti-cancer therapy as a vaccine for inducing an immune response to cells expressing a 55P4H4-related protein. In particular, the generation of anti-idiotypic antibodies is well known in the art; this methodology can readily be adapted to generate anti-idiotypic anti-55P4H4 antibodies that mimic an epitope on a 55P4H4-related protein (see, for example, Wagner et al., 1997, Hybridoma 16: 33-40; Foon et al., 1995, J. Clin. Invest. 96:334-342; Herlyn et al., 1996, Cancer Immunol. Immunother. 43:65-76). Such an anti-idiotypic antibody can be used in cancer vaccine strategies.

#### **XI.) Inhibition of 55P4H4 Protein Function**

The invention includes various methods and compositions for inhibiting the binding of 55P4H4 to its binding partner or its association with other protein(s) as well as methods for inhibiting 55P4H4 function.

##### **XI.A.) Inhibition of 55P4H4 With Intracellular Antibodies**

In one approach, a recombinant vector that encodes single chain antibodies that specifically bind to 55P4H4 are introduced into 55P4H4 expressing cells via gene transfer technologies. Accordingly, the encoded single chain anti-55P4H4 antibody is expressed intracellularly, binds to 55P4H4 protein, and thereby inhibits its function. Methods for engineering such intracellular single chain antibodies are well known. Such intracellular antibodies, also known as "intrabodies", are specifically targeted to a particular compartment within the cell, providing control over where the inhibitory activity of the treatment is focused. This technology has been successfully applied in the art (for review, see Richardson and Marasco, 1995, TIBTECH vol. 13). Intrabodies have been shown to virtually eliminate the expression of otherwise abundant cell surface receptors (see, e.g., Richardson et al., 1995, Proc. Natl. Acad. Sci. USA 92: 3137-3141; Beerli et al., 1994, J. Biol. Chem. 269: 23931-23936; Deshane et al., 1994, Gene Ther. 1: 332-337).

Single chain antibodies comprise the variable domains of the heavy and light chain joined by a flexible linker polypeptide, and are expressed as a single polypeptide. Optionally, single chain antibodies are expressed as a single chain variable region fragment joined to the light chain constant

region. Well-known intracellular trafficking signals are engineered into recombinant polynucleotide vectors encoding such single chain antibodies in order to precisely target the intrabody to the desired intracellular compartment. For example, intrabodies targeted to the endoplasmic reticulum (ER) are engineered to incorporate a leader peptide and, optionally, a C-terminal ER retention signal, such as the KDEL amino acid motif. Intrabodies intended to exert activity in the nucleus are engineered to include a nuclear localization signal. Lipid moieties are joined to intrabodies in order to tether the intrabody to the cytosolic side of the plasma membrane. Intrabodies can also be targeted to exert function in the cytosol. For example, cytosolic intrabodies are used to sequester factors within the cytosol, thereby preventing them from being transported to their natural cellular destination.

In one embodiment, intrabodies are used to capture 55P4H4 in the nucleus, thereby preventing its activity within the nucleus. Nuclear targeting signals are engineered into such 55P4H4 intrabodies in order to achieve the desired targeting. Such 55P4H4 intrabodies are designed to bind specifically to a particular 55P4H4 domain. In another embodiment, cytosolic intrabodies that specifically bind to the 55P4H4 protein are used to prevent 55P4H4 from gaining access to the nucleus, thereby preventing it from exerting any biological activity within the nucleus (e.g., preventing 55P4H4 from forming transcription complexes with other factors).

In order to specifically direct the expression of such intrabodies to particular cells, the transcription of the intrabody is placed under the regulatory control of an appropriate tumor-specific promoter and/or enhancer. In order to target intrabody expression specifically to prostate, for example, the PSA promoter and/or promoter/enhancer can be utilized (See, for example, U.S. Patent No. 5,919,652 issued 6 July 1999).

#### **XI.B.) Inhibition of 55P4H4 with Recombinant Proteins**

In another approach, recombinant molecules bind to 55P4H4 and thereby inhibit 55P4H4 function. For example, these recombinant molecules prevent or inhibit 55P4H4 from accessing/binding to its binding partner(s) or associating with other protein(s). Such recombinant molecules can, for example, contain the reactive part(s) of a 55P4H4 specific antibody molecule. In a particular embodiment, the 55P4H4 binding domain of a 55P4H4 binding partner is engineered into a dimeric fusion protein, whereby the fusion protein comprises two 55P4H4 ligand binding domains linked to the Fc portion of a human IgG, such as human IgG1. Such IgG portion can contain, for example, the C<sub>H</sub>2 and C<sub>H</sub>3 domains and the hinge region, but not the C<sub>H</sub>1 domain. Such dimeric fusion proteins are administered in soluble form to patients suffering from a cancer associated with the expression of 55P4H4, whereby the dimeric fusion protein specifically binds to 55P4H4 and blocks 55P4H4 interaction with a binding partner. Such dimeric fusion proteins are further combined into multimeric proteins using known antibody linking technologies.

#### XI.C.) Inhibition of 5SP4H4 Transcription or Translation

The present invention also comprises various methods and compositions for inhibiting the transcription of the 5SP4H4 gene. Similarly, the invention also provides methods and compositions for inhibiting the translation of 5SP4H4 mRNA into protein.

5 In one approach, a method of inhibiting the transcription of the 5SP4H4 gene comprises contacting the 5SP4H4 gene with a 5SP4H4 antisense polynucleotide. In another approach, a method of inhibiting 5SP4H4 mRNA translation comprises contacting the 5SP4H4 mRNA with an antisense polynucleotide. In another approach, a 5SP4H4 specific ribozyme is used to cleave the 5SP4H4 message, thereby inhibiting translation. Such antisense and ribozyme based methods can also be  
10 directed to the regulatory regions of the 5SP4H4 gene, such as the 5SP4H4 promoter and/or enhancer elements. Similarly, proteins capable of inhibiting a 5SP4H4 gene transcription factor are used to inhibit 5SP4H4 mRNA transcription. The various polynucleotides and compositions useful in the aforementioned methods have been described above. The use of antisense and ribozyme molecules to inhibit transcription and translation is well known in the art.

15 Other factors that inhibit the transcription of 5SP4H4 by interfering with 5SP4H4 transcriptional activation are also useful to treat cancers expressing 5SP4H4. Similarly, factors that interfere with 5SP4H4 processing are useful to treat cancers that express 5SP4H4. Cancer treatment methods utilizing such factors are also within the scope of the invention.

#### XI.D.) General Considerations for Therapeutic Strategies

20 Gene transfer and gene therapy technologies can be used to deliver therapeutic polynucleotide molecules to tumor cells synthesizing 5SP4H4 (i.e., antisense, ribozyme, polynucleotides encoding intrabodies and other 5SP4H4 inhibitory molecules). A number of gene therapy approaches are known in the art. Recombinant vectors encoding 5SP4H4 antisense polynucleotides, ribozymes, factors capable of interfering with 5SP4H4 transcription, and so forth, can be delivered to target tumor cells using such gene  
25 therapy approaches.

The above therapeutic approaches can be combined with any one of a wide variety of surgical, chemotherapy or radiation therapy regimens. The therapeutic approaches of the invention can enable the use of reduced dosages of chemotherapy (or other therapies) and/or less frequent administration, an advantage for all patients and particularly for those that do not tolerate the toxicity of the chemotherapeutic  
30 agent well.

The anti-tumor activity of a particular composition (e.g., antisense, ribozyme, intrabody), or a combination of such compositions, can be evaluated using various *in vitro* and *in vivo* assay systems. *In vitro* assays that evaluate therapeutic activity include cell growth assays, soft agar assays and other assays indicative of tumor promoting activity, binding assays capable of determining the extent to which a  
35 therapeutic composition will inhibit the binding of 5SP4H4 to a binding partner, etc.

*In vivo*, the effect of a 55P4H4 therapeutic composition can be evaluated in a suitable animal model. For example, xenogenic prostate cancer models can be used, wherein human prostate cancer explants or passaged xenograft tissues are introduced into immune compromised animals, such as nude or SCID mice (Klein et al., 1997, Nature Medicine 3: 402-408). For example, PCT Patent Application  
5 WO98/16628, Sawyers et al., published April 23, 1998, describes various xenograft models of human prostate cancer capable of recapitulating the development of primary tumors, micrometastasis, and the formation of osteoblastic metastases characteristic of late stage disease. Efficacy can be predicted using assays that measure inhibition of tumor formation, tumor regression or metastasis, and the like.

*In vivo* assays that evaluate the promotion of apoptosis are useful in evaluating therapeutic  
10 compositions. In one embodiment, xenografts from tumor bearing mice treated with the therapeutic composition can be examined for the presence of apoptotic foci and compared to untreated control xenograft-bearing mice. The extent to which apoptotic foci are found in the tumors of the treated mice provides an indication of the therapeutic efficacy of the composition.

The therapeutic compositions used in the practice of the foregoing methods can be formulated  
15 into pharmaceutical compositions comprising a carrier suitable for the desired delivery method. Suitable carriers include any material that when combined with the therapeutic composition retains the anti-tumor function of the therapeutic composition and is generally non-reactive with the patient's immune system. Examples include, but are not limited to, any of a number of standard pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and the like (see, generally,  
20 Remington's Pharmaceutical Sciences 16<sup>th</sup> Edition, A. Osal., Ed., 1980).

Therapeutic formulations can be solubilized and administered via any route capable of delivering the therapeutic composition to the tumor site. Potentially effective routes of administration include, but are not limited to, intravenous, parenteral, intraperitoneal, intramuscular, intratumor, intradermal, intraorgan, orthotopic, and the like. A preferred formulation for intravenous injection  
25 comprises the therapeutic composition in a solution of preserved bacteriostatic water, sterile unpreserved water, and/or diluted in polyvinylchloride or polyethylene bags containing 0.9% sterile Sodium Chloride for Injection, USP. Therapeutic protein preparations can be lyophilized and stored as sterile powders, preferably under vacuum, and then reconstituted in bacteriostatic water (containing for example, benzyl alcohol preservative) or in sterile water prior to injection.

30 Dosages and administration protocols for the treatment of cancers using the foregoing methods will vary with the method and the target cancer, and will generally depend on a number of other factors appreciated in the art.

## XII.) Kits

For use in the diagnostic and therapeutic applications described herein, kits are also within the  
35 scope of the invention. Such kits can comprise a carrier, package or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the

container(s) comprising one of the separate elements to be used in the method. For example, the container(s) can comprise a probe that is or can be detectably labeled. Such probe can be an antibody or polynucleotide specific for a 55P4H4-related protein or a 55P4H4 gene or message, respectively. Where the method utilizes nucleic acid hybridization to detect the target nucleic acid, the kit can also have containers containing nucleotide(s) for amplification of the target nucleic acid sequence and/or a container comprising a reporter-means, such as a biotin-binding protein, such as avidin or streptavidin, bound to a reporter molecule, such as an enzymatic, florescent, or radioisotope label. The kit can include all or part of the amino acid sequence of Figure 2 or analogs thereof, or a nucleic acid molecule that encodes such amino acid sequences.

10 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

15 A label can be present on the container to indicate that the composition is used for a specific therapy or non-therapeutic application, and can also indicate directions for either *in vivo* or *in vitro* use, such as those described above. Directions and or other information can also be included on an insert which is included with the kit.

20 The 55P4H4 cDNA has been deposited under the requirements of the Budapest Treaty on May 19, 2000, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 USA, as plasmid p55P4H4-EBB12, and has been assigned Accession No. PTA-1894.

### **EXAMPLES**

25 Various aspects of the invention are further described and illustrated by way of the several examples that follow, none of which are intended to limit the scope of the invention.

#### **Example 1: SSH-Generated Isolation of a cDNA Fragment of the 55P4H4 Gene**

30 The SSH cDNA fragment 55P4H4 (Figure 1) was derived from a subtraction experiment where LAPC-4AD (grown intra-tibially in SCID mouse bone) was subtracted from cDNA derived from LAPC-4AD (grown subcutaneously in SCID mice).

#### **Materials and Methods**

##### **LAPC Xenografts and Human Tissues:**

35 LAPC xenografts were obtained from Dr. Charles Sawyers (UCLA) and generated as described (Klein et al, 1997, Nature Med. 3: 402-408; Craft et al., 1999, Cancer Res. 59: 5030-5036). Androgen dependent and independent LAPC-4 xenografts LAPC-4 AD and AI, respectively) and



LAPC-9 AD and AI xenografts were grown in male SCID mice and were passaged as small tissue chunks in recipient males. LAPC-4 and -9 AI xenografts were derived from LAPC-4 or -9 AD tumors, respectively. To generate the AI xenografts, male mice bearing AD tumors were castrated and maintained for 2-3 months. After the tumors re-grew, the tumors were harvested and passaged in castrated males or in female SCID mice.

#### Cell Lines:

Human cell lines (e.g., HeLa) were obtained from the ATCC and were maintained in DMEM with 5% fetal calf serum.

#### RNA Isolation:

Tumor tissue and cell lines were homogenized in Trizol reagent (Life Technologies, Gibco BRL) using 10 ml/ g tissue or 10 ml/ 10<sup>8</sup> cells to isolate total RNA. Poly A RNA was purified from total RNA using Qiagen's Oligotex mRNA Mini and Midi kits. Total and mRNA were quantified by spectrophotometric analysis (O.D. 260/280 nm) and analyzed by gel electrophoresis.

#### Oligonucleotides:

The following HPLC purified oligonucleotides were used.

##### DPNCDN (cDNA synthesis primer):

5'TTTTGATCAAGCTT<sub>30</sub>3' (SEQ ID NO: 10)

##### Adaptor 1:

5'CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGCAG3' (SEQ ID NO: 11)

3'GGCCCGTCCTAG5' (SEQ ID NO: 12)

##### Adaptor 2:

5'GTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAG3' (SEQ ID NO:13)

3'CGGCTCCTAG5' (SEQ ID NO: 14)

##### PCR primer 1:

5'CTAATACGACTCACTATAGGGC3' (SEQ ID NO: 15)

##### Nested primer (NP)1:

5'TCGAGCGGCCGCCCCGGGCAGGA3' (SEQ ID NO: 16)

##### Nested primer (NP)2:

5'AGCGTGGTCGCGGCCGAGGA3'

(SEQ ID NO: 17)

#### Suppression Subtractive Hybridization:

5       Suppression Subtractive Hybridization (SSH) was used to identify cDNAs corresponding to genes that may be differentially expressed in prostate cancer. The SSH reaction utilized cDNA from two LAPC-4 AD (androgen-dependent) xenografts. Specifically, the 55P4H4 SSH sequence was identified from a subtraction where cDNA derived from LAPC-4 AD grown in the tibia (intratibially) was subtracted from cDNA derived from an LAPC-4 AD tumor grown subcutaneously. The SSH DNA sequence of 300 bp (Figure 1) was identified.

10       The LAPC-4 AD xenograft grown sub-cutaneously in SCID mice was used as the source of the "tester" cDNA, while the cDNA from the LAPC-4 AD xenograft grown intratibially was used as the source of the "driver" cDNA. Double stranded cDNAs corresponding to tester and driver cDNAs were synthesized from 2 µg of poly(A)<sup>+</sup> RNA isolated from the relevant xenograft tissue, as described above, using CLONTECH's PCR-Select cDNA Subtraction Kit and 1 ng of oligonucleotide DPNCDN  
15 as primer. First- and second-strand synthesis were carried out as described in the Kit's user manual protocol (CLONTECH Protocol No. PT1117-1, Catalog No. K1804-1). The resulting cDNA was digested with Dpn II for 3 hrs at 37°C. Digested cDNA was extracted with phenol/chloroform (1:1) and ethanol precipitated.

20       Driver cDNA was generated by combining in a 1:1 ratio Dpn II digested cDNA from the relevant xenograft source (see above) with a mix of digested cDNAs derived from benign prostate hyperplasia (BPH) and the human cell lines HeLa, 293, A431, Colo205, and mouse liver.

25       Tester cDNA was generated by diluting 1 µl of Dpn II digested cDNA from the relevant xenograft source (see above) (400 ng) in 5 µl of water. The diluted cDNA (2 µl, 160 ng) was then ligated to 2 µl of Adaptor 1 and Adaptor 2 (10 µM), in separate ligation reactions, in a total volume of 10 µl at 16°C overnight, using 400 u of T4 DNA ligase (CLONTECH). Ligation was terminated with 1 µl of 0.2 M EDTA and heating at 72°C for 5 min.

30       The first hybridization was performed by adding 1.5 µl (600 ng) of driver cDNA to each of two tubes containing 1.5 µl (20 ng) Adaptor 1- and Adaptor 2- ligated tester cDNA. In a final volume of 4 µl, the samples were overlaid with mineral oil, denatured in an MJ Research thermal cycler at 98°C for 1.5 minutes, and then were allowed to hybridize for 8 hrs at 68°C. The two hybridizations were then mixed together with an additional 1 µl of fresh denatured driver cDNA and were allowed to hybridize overnight at 68°C. The second hybridization was then diluted in 200 µl of 20 mM Hepes, pH 8.3, 50 mM NaCl, 0.2 mM EDTA, heated at 70°C for 7 min. and stored at -20°C.

#### PCR Amplification, Cloning and Sequencing of Gene Fragments Generated from SSH:

To amplify gene fragments resulting from SSH reactions, two PCR amplifications were performed. In the primary PCR reaction 1  $\mu$ l of the diluted final hybridization mix was added to 1  $\mu$ l of PCR primer 1 (10  $\mu$ M), 0.5  $\mu$ l dNTP mix (10  $\mu$ M), 2.5  $\mu$ l 10 x reaction buffer (CLONTECH) and 5 0.5  $\mu$ l 50 x Advantage cDNA polymerase Mix (CLONTECH) in a final volume of 25  $\mu$ l. PCR 1 was conducted using the following conditions: 75°C for 5 min., 94°C for 25 sec., then 27 cycles of 94°C for 10 sec, 66°C for 30 sec, 72°C for 1.5 min. Five separate primary PCR reactions were performed for each experiment. The products were pooled and diluted 1:10 with water. For the secondary PCR reaction, 1  $\mu$ l from the pooled and diluted primary PCR reaction was added to the same reaction mix as 10 used for PCR 1, except that primers NP1 and NP2 (10  $\mu$ M) were used instead of PCR primer 1. PCR 2 was performed using 10-12 cycles of 94°C for 10 sec, 68°C for 30 sec, and 72°C for 1.5 minutes. The PCR products were analyzed using 2% agarose gel electrophoresis.

The PCR products were inserted into pCR2.1 using the T/A vector cloning kit (Invitrogen). Transformed *E. coli* were subjected to blue/white and ampicillin selection. White colonies were picked 15 and arrayed into 96 well plates and were grown in liquid culture overnight. To identify inserts, PCR amplification was performed on 1 ml of bacterial culture using the conditions of PCR1 and NP1 and NP2 as primers. PCR products were analyzed using 2% agarose gel electrophoresis.

Bacterial clones were stored in 20% glycerol in a 96 well format. Plasmid DNA was prepared, sequenced, and subjected to nucleic acid homology searches of the GenBank, dBest, and 20 NCI-CGAP databases.

#### RT-PCR Expression Analysis:

First strand cDNAs were generated from 1  $\mu$ g of mRNA with oligo (dT)12-18 priming using the Gibco-BRL Superscript Preamplification system. The manufacturer's protocol was used which 25 included an incubation for 50 min at 42°C with reverse transcriptase followed by RNase H treatment at 37°C for 20 min. After completing the reaction, the volume was increased to 200  $\mu$ l with water prior to normalization.

Normalization of the first strand cDNAs from multiple normal and cancer tissues was performed by using  $\beta$ -actin primers. First strand cDNA (5  $\mu$ l) were amplified in a total volume of 50 30  $\mu$ l containing 0.4  $\mu$ M primers, 0.2  $\mu$ M each dNTPs, 1XPCR buffer (Gibco-BRL, 10 mM Tris-HCL, 1.5 mM  $MgCl_2$ , 50 mM KCl, pH8.3) and 1X Platinum Taq DNA polymerase (Gibco-BRL). PCR was performed using an MJ Research thermal cycler under the following conditions: Initial denaturation can be at 94°C for 45 sec, followed by a 18, 20, and 22 cycles of 94°C for 45, 58°C for 45 sec, 72°C for 45 sec. A final extension at 72°C was carried out for 2 min. Five  $\mu$ l of the PCR reaction were 35 removed at 18, 20, and 22 cycles and used for agarose gel electrophoresis. After agarose gel

electrophoresis, the band intensities of the 283 b.p.  $\beta$ -actin bands from multiple tissues were compared by visual inspection. Dilution factors for the first strand cDNAs were calculated to result in equal  $\beta$ -actin band intensities in all tissues after 22 cycles of PCR. Three rounds of normalization can be required to achieve equal band intensities in all tissues after 22 cycles of PCR.

5 To determine expression levels of the gene, 5  $\mu$ l of normalized first strand cDNA were analyzed by PCR using 26, and 30 cycles of amplification. Semi-quantitative expression analysis was achieved by comparing the PCR products at cycle numbers that give light band intensities. RT-PCR expression analysis was performed on first strand cDNAs generated using pools of tissues from multiple normal and cancer samples. The cDNA normalization was demonstrated in every experiment  
10 using beta-actin PCR.

#### Example 2: Full Length Cloning of 55P4H4 and Homology Comparison to Known Sequences

A full length 55P4H4 cDNA clone (clone EBE12) of 2610 base pairs (bp) was cloned from a skeletal muscle cDNA library (FIG. 2). The cDNA encodes a putative open reading frame (ORF) of  
15 193 amino acid protein with calculated molecular weight of 21.7kDa, and pI of 7.4. 55P4H4 is predicted to be a soluble cytoplasmic protein (52.2%), with a slight possibility of mitochondrial (21.7%) or nuclear (21.7%) localization by PSORT analysis..

Sequence analysis of 55P4H4 reveals homology to a protein that is regulated by hypoxia (PCT/US98/17296, WO 99/09049). The 55P4H4 ORF is 32% identical and 55% homologous to RTP779 over a 180 amino acid region, and 32% identical and 54% homologous to RTP801, the rat orthologue of RTP779 (Fig. 4). 55P4H4 is predicted to be a cytoplasmic protein by PSORT analysis (<http://psort.ims-u-tokyo.ac.jp/form.html>) with a lower possibility of nuclear or mitochondrial localization.

Homology to hypoxia regulated genes provides evidence that 55P4H4 is also regulated by  
25 hypoxia. Most malignant tumors exhibit a low oxygen tension. This may be due to a rate of cellular proliferation that outpaces angiogenesis and a defective tumor microcirculation. However, tumor progression to a lethal phenotype is associated with increased adaptation to hypoxia (Semenza, 1999, Ann. Rev. Cell Dev. Biol. 15:551). This adaptive response includes an induction of angiogenesis and an increased metabolic rate. The primary gene responsible for the adaptive response of tumors to  
30 hypoxia is Hypoxia-Inducible Factor 1 (HIF-1), a heterodimeric basic-helix-loop-helix-PAS transcription factor (Semenza, 1999, Ann. Rev. Cell Dev. Biol. 15:551). HIF-1 consists of an  $\alpha$  and a  $\beta$  subunit. The  $\beta$  subunit is constitutively expressed, while the  $\alpha$  subunit is regulated by hypoxia (Semenza, 1999, Ann. Rev. Cell Dev. Biol. 15:551; Gustafsson et al., 1999, Am. J. Physiol. 276(2 pt 2): H679-685). HIF-1 $\alpha$  overexpression has been detected in human tumors, including prostate cancer  
35 (Zhong et al., 1999, Cancer Res. 59:5830-5835). As angiogenesis is a complex process involving a

variety of processes, other genes, such as 55P4H4 and related molecules, may contribute to the adaptive response to hypoxia (Hanahan et al., Cell 1996 86(3):353-64). As 55P4H4 exhibits one potential nuclear localization signal it may as a transcription factor regulating angiogenic genes and genes involved in glycolysis. HIF-1 has been shown to regulate the genes encoding erythropoietin (EPO), vascular-endothelial growth factor (VEGF), glycolytic enzymes, and the glucose transporter which are transactivated by HIF-1 (Gleadle and Ratcliffe, 1997, Blood 89:503-509; Semenza, 1999, Ann. Rev. Cell Dev. Biol. 15:551).

Among normal tissues 55P4H4 expression has been detected predominantly in skeletal muscle, a tissue with a high metabolic rate. HIF-1 contributes to aerobic glycolysis in tumors, an early sign of cellular proliferation. 55P4H4 may contribute to regulate metabolic pathways in normal skeletal muscle and in prostate tumors. It may also be that 55P4H4, like HIF-1, may be regulated by oncogene activation and/or tumor suppressor inactivation (Semenza, 1999, Ann. Rev. Cell Dev. Biol. 15:551).

Inhibition of the hypoxia signaling pathway would block an essential step in the growth of solid tumors and their metastasis. Such potential therapies are contemplated for HIF-1 (Blancher and Harris, 1998, Cancer Metastasis Rev. 17:187-194). 55P4H4 expression is induced in several cell lines and xenografts derived from solid tumors, including tumors of the prostate, bladder, brain, bone, lung, kidney, testis and ovaries. It is, therefore, a potential target for small molecule and vaccine therapeutics in those types of cancers.

As disclosed herein, 55P4H4 function can be assessed in mammalian cells. Cells and tumors that overexpress 55P4H4 can be tested for angiogenic activity using in vitro and/or in vivo assays. The 55P4H4 cell phenotype can be compared to the phenotype of cells that lack expression of 55P4H4. Experiments can be performed to determine whether overexpression of 55P4H4 induces the expression of angiogenic factors and/or glycolytic/metabolic pathway factors. The relationship of HIF-1 $\alpha$  to 55P4H4 can be investigated by determining whether overexpression of one gene will induce expression of the other gene. In addition, cells can be manipulated to grow under hypoxic conditions to evaluate and compare the expression of both genes.

As shown in Figure 5, 55P4H4 shows homology to a mouse protein RIK of unknown function (85% identity). As shown in Figure 5, the 55P4H4 protein shows distinct homology to yeast RIC-1 protein, with 27% identity and 47% homology, as well as the Drosophila CHARYBDE protein, with 33% identity and 49% homology.

RIC-1 has been shown to be involved in the transcription of ribosomal RNA and synthesis of ribosomal protein (Mizuta K, et al. Gene 1997. 187:171). In addition, Ric-1 is involved in cellular trafficking and localization of trans-golgi proteins (Bensen ES, Yeung BG, Payne GS. Mol Biol Cell. 2001 12:13). Charybde is a Homeotic Complex (Hox) target protein identified in Drosophila melanogaster (Chauvet, S et al). Hox genes regulate cell fate decisions, such as development and

differentiation by regulating the transcription of several genes (Volpe MV, Vosatka RJ, Nielsen HC. Biochim Biophys Acta. 2000, 26;1475; Alper S, Kenyon C. Development. 2001, 128:1793; Fuller FJ et al. Blood. 1999, 93:3391). Since several Hox-regulated genes have been associated with cancer, 55P4H4 may function as a Hox effector and participate in the regulation of tumor progression (Kros J, Sauvageau G, Oncogene. 2000, 19:5134).

The 55P4H4 cDNA has been deposited under the requirements of the Budapest Treaty on May 19, 2000, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 USA, as plasmid p55P4H4-EBB12, and has been assigned Accession No. PTA-1894.

### 10 Example 3: Chromosomal Mapping of the 55P4H4 Gene

The chromosomal localization of 55P4H4 was determined using the GeneBridge4 radiation hybrid panel (Walter et al., 1994, Nat. Genetics 7:22; Research Genetics, Huntsville, AL). The following PCR primers were used to localize 55P4H4:

15 55P4H4.1 5' TAGCTGCAGTTGCTATGAATGTGA 3' (SEQ ID NO: 18)  
55P4H4.2 5' CTCAGCTCAGGATTTCGACTTGTT 3' (SEQ ID NO: 19)

The resulting mapping vector for the radiation hybrid panel DNAs was:

20 01000011010000000110010101001101000001001001010000100000100001000100000  
011000002010100001011

Ins  
Bg } 25 This vector and the mapping program at <http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl> placed 55P4H4 to chromosome 4q22.3-24. A variety of chromosomal abnormalities in 4q22.3-24 including amplifications have been identified as frequent cytogenetic abnormalities in a number of different cancers. Nilbert et al., 1988, Cancer Genet. Cytogenet. 34(2): 209-218; Yeatman et al., 1996, Clin. Exp. Metastasis 14(3):246-252; and Joos et al., 2000, Cancer Res. 60(3): 549-552.

### 30 Example 4: Expression analysis of 55P4H4 in normal tissues, cancer cell lines and patient samples

55P4H4 mRNA expression in normal human tissues was analyzed by Northern blotting of two multiple tissue blots (Clontech; Palo Alto, California), comprising a total of 16 different normal human tissues, using labeled 55P4H4 SSH fragment (Example 1) as a probe. RNA samples were 35 quantitatively normalized with a  $\beta$ -actin probe. The 55P4H4 gene produces a transcript of approximately 3.0-3.5 kb. The results of the Northern of 16 different normal human tissues demonstrated expression in skeletal muscle and, at a much lower level, in kidney (FIG. 6).

To analyze 55P4H4 expression in prostate cancer tissues, northern blotting was performed on RNA derived from the LAPC xenografts (Fig. 6). The results show very high expression levels of the transcript in LAPC-4 AD which provides evidence that 55P4H4 is up-regulated in prostate cancer. To further analyze 55P4H4 expression in cancer tissues northern blotting was performed on RNA derived from prostate cancer xenografts grown subcutaneously (sc) or intra-tibially (it) within the mouse bone (Fig. 7). This analysis shows the up-regulation of 55P4H4 expression in bone growing prostate cancer tumors.

To analyze 55P4H4 expression in various cancer tissues, northern blotting was performed on RNA derived from several prostate and non-prostate cancer cell lines. The results show that, in addition to the LAPC-xenografts, the 55P4H4 transcript was detected in several cancer cell lines derived from prostate (DU145, LNCaP), bladder (TCCSUP, 5637), testis (NCCIT, TERA-1, TERA-2), cervix (HeLa), ovary (OV-1063, PA-1, SW626) brain (PFSK-1), bone (SK-ES-1, HOS, RD-ES), lung (NEI-H82), kidney (CAKI-1, SW839) (FIG. 8). Northern analysis also shows that 55P4H4 is expressed in the normal prostate and prostate tumor tissues derived from prostate cancer patients (FIG. 9). The detection of 55P4H4 expression in the normal adjacent tissue of the prostate cancer patients may be due to the presence of cancer glands within the normal tissue. Alternatively, an up-regulation of 55P4H4 may manifest itself early in the disease, even in the seemingly normal part of the prostate.

Figure 10 shows the results of RT-PCR analysis of 55P4H4 expression in various pooled cancer tissues after 30 cycles. 55P4H4 expression was observed in each of the following tissues: xenograft pool (lane 1), prostate cancer pool (lane 2), lung cancer pool (lane 3), ovarian cancer pool (lane 4), Lane 5 is a water blank.

These results suggest that 55P4H4 is generally up-regulated in cancer cells and cancer tissues, especially from prostate cancer, and provides a suitable target for cancer therapy.

#### **Example 5: Amino Acid Scale Profiles and Identification of Antigenic Regions of 55P4H4**

Figures 11, 12, 13, 14, and 15 depict graphically five amino acid profiles of the 55P5H4 amino acid sequence, each assessment available by accessing the ProtScale website (<http://www.expasy.ch/cgi-bin/protscale.pl>) on the Expasy molecular biology server.

These profiles: Figure 11, Hydrophilicity, (Hopp T.P., Woods K.R., 1981. Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828), Figure 12, Hydropathicity, (Kyte J., Doolittle R.F., 1982. J. Mol. Biol. 157:105-132); Figure 13, Percentage Accessible Residues (Janin J., 1979 Nature 277:491-492), Figure 14, Average Flexibility, (Bhaskaran R., and Ponnuswamy P.K., 1988. Int. J. Pept. Protein Res. 32:242-255); Figure 15, Beta-turn (Deleage, G., Roux B. 1987 Protein Engineering 1:289-294); and optionally others on the ProtScale website, were used to identify antigenic regions of the 55P5H4 protein. The above amino acid profiles of 55P5H4 were generated using the following ProtScale parameters for analysis: 1) a window size of 9; 2) 100% weight of the window edges compared to the window center; and, 3) amino acid profile values normalized to lie between 0 and 1.

Hydrophilicity (Figure 11), Hydropathicity (Figure 12) and Percentage Accessible Residues (Figure 13) profiles are used to determine stretches of hydrophilic amino acids (i.e., values greater than 0.5 on the Hydrophilicity and Percentage Accessible Residues profile, and values less than 0.5 on the Hydropathicity profile). Such regions are likely to be exposed to the aqueous environment, present on the surface of the protein, and thus available for immune recognition.

Average Flexibility (Figure 14) and Beta-turn (Figure 15) profiles determine stretches of amino acids (i.e., values greater than 0.5 on the Beta-turn profile and Average Flexibility profile) that are not constrained in secondary structures such as beta sheets and alpha helices. Such regions are also more likely to be exposed on the protein and thus accessible to immune recognition.

Antigenic sequences of the 55P5H4 protein indicated, e.g., by the profiles set forth in Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15 are used to design immunogens, peptides or nucleic acids that encode them, to generate therapeutic and diagnostic anti-55P5H4 antibodies.

#### **Example 6: Generation of 55P5H4 Polyclonal Antibodies**

Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. In addition to immunizing with the full length 55P5H4 protein, computer algorithms are employed in design of immunogens that based on amino acid sequence analysis contain characteristics of being antigenic and available for recognition by the immune system of the immunized host (see, e.g., the Example entitled "Antigenicity Profiles"). Such regions are predicted to be hydrophilic, flexible, in beta-turn conformations, and be exposed on the surface of the protein (see, e.g., Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15 for amino acid profiles that predict such regions of 55P5H4).

For example, 55P5H4 recombinant bacterial fusion proteins or peptides encoding hydrophilic, flexible, beta-turn regions of the 55P5H4 sequence, such as amino acids 62-75 of 55P5H4 are used to immunize New Zealand White rabbits. The peptide can be conjugated to keyhole limpet hemocyanin (KLH) and used to immunize the rabbit. Alternatively the immunizing agent may include all or portions of the 55P5H4 protein, analogs or fusion proteins thereof. For example, the 55P5H4 amino acid sequence can be fused to any one of a variety of fusion protein partners that are well known in the art, such as maltose binding protein, LacZ, thioredoxin or an immunoglobulin constant region (see e.g. Current Protocols In Molecular Biology, Volume 2, Unit 16, Frederick M. Ausubul et al. eds., 1995; Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L., Damle, N., and Ledbetter, L.(1991) J.Exp. Med. 174, 561-566). Other recombinant bacterial proteins include glutathione-S-transferase (GST), and HIS tagged fusion proteins of 55P5H4 that are purified from induced bacteria using the appropriate affinity matrix.



It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).

In a typical protocol, rabbits are initially immunized subcutaneously with up to 200 µg, typically 100-200 µg, of fusion protein or peptide conjugated to KLH mixed in complete Freund's adjuvant. Rabbits are then injected subcutaneously every two weeks with up to 200 µg, typically 100-200 µg, of immunogen in incomplete Freund's adjuvant. Test bleeds are taken approximately 7-10 days following each immunization and used to monitor the titer of the antiserum by ELISA.

To test serum, such as rabbit serum, for reactivity with 55P5H4 proteins, the full-length 55P5H4 cDNA can be cloned into an expression vector such as one that provides a 6His tag at the carboxyl-terminus (pCDNA 3.1 myc-his, Invitrogen, see Example 8). After transfection of the constructs into 293T cells, cell lysates can be probed with anti-His antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and the anti-55P5H4 serum using Western blotting. Alternatively specificity of the antiserum is tested by Western blot and immunoprecipitation analyses using lysates of cells that express 55P5H4. Serum from rabbits immunized with GST or MBP fusion proteins is first semi-purified by removal of anti-GST or anti-MBP antibodies by passage over GST and MBP protein columns respectively. Sera from His-tagged protein and peptide immunized rabbits as well as depleted GST and MBP protein sera are purified by passage over an affinity column composed of the respective immunogen covalently coupled to Affigel matrix (BioRad).

#### **Example 7: Generation of 55P5H4 Monoclonal Antibodies (mAbs)**

In one embodiment, therapeutic mAbs to 55P5H4 will include those that react with epitopes of the protein that would disrupt or modulate the biological function of 55P5H4. Immunogens for generation of such mAbs are designed to encode or contain the entire 55P5H4 protein or regions of the 55P5H4 protein predicted to be antigenic from computer analysis of the amino acid sequence (see, e.g., Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15). Additionally, regions or amino acid sequences that encode, contain, or have homology to known functional motifs such as those indicated in Tables V-XX are useful as immunogens for generation of therapeutic antibodies. These immunogens include peptides, recombinant bacterial proteins, and mammalian expressed Tag5 proteins and human and murine IgG FC fusion proteins. To generate mAbs to 55P5H4, mice are first immunized intraperitoneally (IP) with typically 10-50 µg, of protein immunogen mixed in complete Freund's adjuvant. Mice are then subsequently immunized IP every 2-4 weeks with typically 10-50 µg, of antigen mixed in Freund's incomplete adjuvant. Alternatively, Ribi adjuvant is used

immunizations. In addition, a DNA-based immunization protocol is employed in which a mammalian expression vector encoding 55P5H4 sequence is used to immunize mice by direct injection of the plasmid DNA. For example, pCDNA 3.1 encoding the full length 55P5H4 cDNA fused at the N-terminus to an IgK leader sequence and at the C-terminus to the coding sequence of murine or human IgG is used. This protocol is used alone or in combination with protein immunogens. Test bleeds are taken 7-10 days following immunization to monitor titer and specificity of the immune response. Once appropriate reactivity and specificity is obtained as determined by ELISA, Western blotting, and immunoprecipitation analyses, fusion and hybridoma generation is then carried with established procedures well known in the art (Harlow and Lane, 1988).

In one embodiment for generating 55P5H4 monoclonal antibodies, a glutathione-S-transferase (GST) fusion protein encoding the full length 55P5H4 protein is expressed, purified, and used as immunogen. Balb C mice are initially immunized intraperitoneally with 25 µg of the GST-55P5H4 fusion protein mixed in complete Freund's adjuvant. Mice are subsequently immunized every two weeks with 25 µg of GST-55P5H4 protein mixed in Freund's incomplete adjuvant for a total of three immunizations. To determine titer of serum from immunized mice, ELISA is carried out using a 55P5H4-specific cleavage fragment of the immunogen in which GST is removed by site specific proteolysis. Reactivity and specificity of serum to full length 55P5H4 protein is monitored by Western blotting, immunoprecipitation, and flow cytometry using 293T cells transfected with an expression vector encoding the 55P5H4 cDNA (see, e.g., Example 8). Mice showing the strongest reactivity are rested for three weeks and given a final injection of 55P5H4 cleavage fragment in PBS and then sacrificed four days later. The spleens of the sacrificed mice are then harvested and fused to SPO/2 myeloma cells using standard procedures (Harlow and Lane, 1988). Supernatants from growth wells following HAT selection are screened by ELISA, Western blot, flow cytometry, and immunoprecipitation, to identify 55P5H4 specific antibody-producing clones.

The binding affinity of a 55P5H4 monoclonal antibody is determined using standard technologies. Affinity measurements quantify the strength of antibody to epitope binding and can be used to help define which 55P5H4 monoclonal antibodies are preferred for diagnostic or therapeutic use. The BIAcore system (Uppsala, Sweden) is a preferred method for determining binding affinity. The BIAcore system uses surface plasmon resonance (SPR, Welford K. 1991, Opt. Quant. Elect. 23:1; Morton and Myszk, 1998, Methods in Enzymology 295: 268) to monitor biomolecular interactions in real time. BIAcore analysis conveniently generates association rate constants, dissociation rate constants, equilibrium dissociation constants, and affinity constants.

#### **Example 8: Production of Recombinant 55P4H4 in Bacterial and Mammalian Constructs**

## BACTERIAL CONSTRUCTS

### pGEX Constructs

To express 55P4H4 in bacterial cells, cDNA coding for the 193 amino acid ORF or partial length 55P4H4 cDNA (such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof) was fused to the Glutathione S-transferase (GST) gene by cloning into pGEX-6P-1 (Amersham Pharmacia Biotech, NJ). The construct was made in order to generate recombinant 55P4H4 protein sequences with GST fused at the N-terminus and a six histidine epitope at the C-terminus. The six histidine epitope tag is generated by adding the histidine codons to the cloning primer at the 3' end of the open reading frame (ORF). A PreScission™ recognition site permits cleavage of the GST tag from 55P4H4-related protein. The ampicillin resistance gene and pBR322 origin permits selection and maintenance of the plasmid in *E. coli*. Alternatively, partial length 55P4H4 cDNA (such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof) is fused to the Glutathione S-transferase (GST) gene.

### pMAL Constructs

To express 55P4H4 in bacterial cells, all or part of the 55P4H4 nucleic acid sequence (such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof) are fused to the maltose-binding protein (MBP) gene by cloning into pMAL-c2X and pMAL-p2X (New England Biolabs, MA). The constructs are made to generate recombinant 55P4H4 protein sequences with MBP fused at the N-terminus and a six histidine epitope at the C-terminus. The six histidine epitope tag is generated by adding histidine codons to the 3' cloning primer. A Factor Xa recognition site permits cleavage of the GST tag from 55P4H4. The pMAL-c2X and pMAL-p2X vectors are optimized to express the recombinant protein in the cytoplasm or periplasm respectively. Periplasm expression enhances folding of proteins with disulfide bonds.

### pCRII

To generate 55P4H4 sense and anti-sense riboprobes for RNA *in situ* investigations, a pCRII construct (Invitrogen, Carlsbad CA) is generated using cDNA sequence encoding the ORF or fragments of the cDNA (such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof). The pCRII vector has Sp6 and T7 promoters flanking the insert to drive the production of 55P4H4 RNA riboprobes for use in RNA *in situ* hybridization experiments.

## MAMMALIAN CONSTRUCTS

To express recombinant 55P4H4, the full or partial length 55P4H4 cDNA (such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof) can be cloned into any one of a variety of expression vectors known in the art. The constructs can be transfected into any one of a wide variety of mammalian cells such as 293T cells. Transfected 293T cell lysates can be probed with anti-55P4H4 polyclonal serum in a Western blot experiment.

The 55P4H4 gene and cDNA fragments can also be subcloned into the retroviral expression vector pSR $\alpha$ MSVtkneo and used to establish 55P4H4-expressing cell lines as follows: The 55P4H4 coding sequence (from translation initiation ATG and Kozak translation start consensus sequence to the termination codons) is amplified by PCR using the 55P4H4 cDNA. The PCR product is subcloned into pSR $\alpha$ MSVtkneo vector and transformed into DH5 $\alpha$  competent cells. Colonies are picked to screen for clones with unique internal restriction sites on the cDNA. The positive clone is confirmed by sequencing of the cDNA insert. The retroviral vectors can thereafter be used for infection and generation of various cell lines using, for example, NIH 3T3, TsuPr1, 293 or rat-1 cells.

Additional illustrative mammalian systems are discussed below.

### pcDNA4/HisMax-TOPO Constructs

To express 55P4H4 in mammalian cells, the 55P4H4 ORF (or a portion thereof such as that encoding amino acids 1 to 193 or any 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 continuous amino acids from 55P4H4 or an analog thereof) is cloned into pcDNA4/HisMax-TOPO Version A (cat# K864-20, Invitrogen, Carlsbad, CA). Protein expression is driven from the cytomegalovirus (CMV) promoter and the SP163 translational enhancer. The recombinant protein has Xpress<sup>TM</sup> and six histidine epitopes fused to the N-terminus. The pcDNA4/HisMax-TOPO vector also contains the bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability along with the SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen. The Zeocin resistance gene allows for selection of mammalian cells expressing the protein and the ampicillin resistance gene and ColE1 origin permits selection and maintenance of the plasmid in E. coli.

### pcDNA3.1/MycHis Constructs

To express 55P4H4 in mammalian cells, the ORF with consensus Kozak translation initiation site was cloned into pcDNA3.1/MycHis\_Version A (Invitrogen, Carlsbad, CA). Also, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are clone into pcDNA3.1/MycHis\_Version A (Invitrogen, Carlsbad, CA). Protein expression is driven from the

cytomegalovirus (CMV) promoter. The recombinant protein has the myc epitope and six histidines fused to the C-terminus. The pcDNA3.1/MycHis vector also contains the bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability, along with the SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen. The Neomycin resistance gene can be used, as it allows for selection of mammalian cells expressing the protein and the ampicillin resistance gene and ColE1 origin permits selection and maintenance of the plasmid in *E. coli*.

#### pcDNA3.1/Vhis-TOPO Construct

To express 55P4H4 in mammalian cells, the cDNA encoding amino acids 1 to 193 is cloned along with Kozak consensus translation initiation sequence into pcDNA4/V5His-TOPO (cat# K4800-01, Invitrogen, Carlsbad, CA). Also, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino-acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into pcDNA4/V5His-TOPO (cat# K4800-01, Invitrogen, Carlsbad, CA). Protein expression is driven from the cytomegalovirus (CMV) promoter. The recombinant protein has V5<sup>TM</sup> and six histidine epitopes fused at the C-terminus to aid in detection and purification of the recombinant protein. The pcDNA4/V5His-TOPO vector also contains the bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability along with the SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen. The Zeocin resistance gene allows for selection of mammalian cells expressing the protein and the ampicillin resistance gene and the ColE1 origin permits selection and maintenance of the plasmid in *E. coli*.

#### pcDNA3.1CT-GFP-TOPO Construct

To express 55P4H4 in mammalian cells and to allow detection of the recombinant protein using fluorescence, the cDNA coding for the ORF with consensus Kozac translation initiation site is cloned into pcDNA3.1CT-GFP-TOPO (Invitrogen, CA). Alternatively, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into pcDNA3.1CT-GFP-TOPO (Invitrogen, CA). Protein expression is driven from the cytomegalovirus (CMV) promoter. The recombinant protein has the Green Fluorescent Protein (GFP) fused to the C-terminus facilitating non-invasive, in vivo detection and cell biology studies. The pcDNA3.1/MycHis vector also contains the bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability along with the SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigen. The Neomycin resistance gene allows for selection of mammalian cells that express the protein, and the ampicillin resistance gene and

ColE1 origin permits selection and maintenance of the plasmid in *E. coli*. An additional construct with a N-terminal GFP fusion is made in pcDNA3.1NT-GFP-TOPO spanning the entire length of the 55P4H4 protein.

#### 5 **pAPtag**

The cDNA coding for the 55P4H4 ORF is cloned into pAPtag-5 (GenHunter Corp. Nashville, TN). This construct generates an alkaline phosphatase fusion at the C-terminus of the 55P4H4 protein while fusing the IgGK signal sequence to N-terminus. Alternatively, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into pAPtag-5 (GenHunter Corp. Nashville, TN). The resulting recombinant 55P4H4 protein is optimized for secretion into the media of transfected mammalian cells and can be used to identify proteins such as ligands or receptors that interact with the 55P4H4-protein. Protein expression is driven from the CMV promoter and the recombinant protein also contains myc and six histidines fused to the C-terminus of alkaline phosphatase. The Zeosin resistance gene allows for selection of mammalian cells expressing the protein and the ampicillin resistance gene permits selection of the plasmid in *E. coli*.

#### **ptag5**

The cDNA coding for the 55P4H4 ORF is also cloned into pTag-5. This vector is similar to pAPtag but without the alkaline phosphatase fusion. This construct fuses the IgGK signal sequence to the N-terminus of inserts. The resulting recombinant 55P4H4 protein is optimized for secretion into the media of transfected mammalian cells, and can be used to identify proteins such as ligands or receptors that interact with the 55P4H4 protein. Protein expression is driven from the CMV promoter and the recombinant protein also contains myc and six histidines fused to the C-terminus of alkaline phosphatase. The Zeosin resistance gene allows for selection of mammalian cells expressing the protein, and the ampicillin resistance gene permits selection of the plasmid in *E. coli*. Alternatively, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into pTag-5.

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#### **psecFc**

The cDNA coding for the 55P4H4 ORF was also cloned into psecFc. The psecFc vector was assembled by cloning immunoglobulin G1 Fc (hinge, CH2, CH3 regions) into pSecTag2 (Invitrogen, California). This construct generates an immunoglobulin G1 Fc fusion at the C-terminus of the 55P4H4 protein, while fusing the IgGK signal sequence to N-terminus. The resulting recombinant 55P4H4 protein is optimized for secretion into the media of transfected mammalian cells, and can be

used to identify proteins such as ligands or receptors that interact with the 55P4H4 protein. Protein expression is driven from the CMV promoter and the recombinant protein also contains myc and six histidines fused to the C-terminus of alkaline phosphatase. The Zeosin resistance gene allows for selection of mammalian cells that express the protein, and the ampicillin resistance gene permits selection of the plasmid in *E. coli*. Alternatively, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into psecFc.

#### pSR $\alpha$ Constructs

To generate mammalian cell lines that express 55P4H4 constitutively, the cDNA coding for the ORF was cloned into pSR $\alpha$  constructs. Amphotropic and ecotropic retroviruses were generated by transfection of pSR $\alpha$  constructs into the 293T-10A1 packaging line or co-transfection of pSR $\alpha$  and a helper plasmid ( $\phi$ ) in the 293 cells, respectively. The retrovirus can be used to infect a variety of mammalian cell lines, resulting in the integration of the cloned gene, 55P4H4, into the host cell lines. Protein expression is driven from a long terminal repeat (LTR). The Neomycin resistance gene allows for selection of mammalian cells that express the protein, and the ampicillin resistance gene and ColE1 origin permit selection and maintenance of the plasmid in *E. coli*. Alternatively, nucleic acids that encode a portion of 55P4H4 such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 contiguous amino acids from 55P4H4 or an analog thereof, along with Kozak consensus translation initiation sequence are cloned into pSR $\alpha$  constructs.

An additional pSR $\alpha$  construct is made that fuses the FLAG tag to the C-terminus to allow detection using anti-FLAG antibodies. The FLAG sequence 5' gat tac aag gat gac gac gat aag 3' (SEQ ID NO: 20) was added to the cloning primer at the 3' end of the ORF, or portion thereof. Additional pSR $\alpha$  constructs are made to produce both N-terminal and C-terminal GFP and myc/6 HIS fusion proteins of the full-length 55P4H4 protein.

#### Example 9: Production of Recombinant 55P4H4 in a Baculovirus System

To generate a recombinant 55P4H4 protein in a baculovirus expression system, cDNA sequence encoding the 55P4H4 protein is cloned into the baculovirus transfer vector pBlueBac 4.5 (Invitrogen), which provides a His-tag at the N-terminus. Specifically, pBlueBac--55P4H4 is co-transfected with helper plasmid pBac-N-Blue (Invitrogen) into SF9 (*Spodoptera frugiperda*) insect cells to generate recombinant baculovirus (see Invitrogen instruction manual for details). Baculovirus is then collected from cell supernatant and purified by plaque assay.

Recombinant 55P4H4 protein is then generated by infection of HighFive insect cells (Invitrogen) with the purified baculovirus. Recombinant 55P4H4 protein can be detected using anti-

55P4H4 antibody. 55P4H4 protein can be purified and used in various cell-based assays or as immunogen to generate polyclonal and monoclonal antibodies specific for 55P4H4.

#### **Example 10: Western Analysis of 55P4H4 Expression in Subcellular Fractions**

- 5           Sequence analysis of 55P4H4 revealed the presence of nuclear localization signal. The cellular location of 55P4H4 can be assessed using subcellular fractionation techniques widely used in cellular biology (Storrie B, et al., 1990, Methods Enzymol. 182:203-25). Prostate or other cell lines can be separated into nuclear, cytosolic and membrane fractions. The expression of 55P4H4 in the different fractions can be tested using western blotting techniques.
- 10           Alternatively, to determine the subcellular localization of 55P4H4, 293T cells can be transfected with an expression vector encoding HIS-tagged 55P4H4 (PCDNA 3.1 MYC/HIS, Invitrogen). The transfected cells can be harvested and subjected to a differential subcellular fractionation protocol as previously described (Pemberton, P.A. et al., 1997, J. Histochem. Cytochem. 45:1697-1706.) This protocol separates the cell into fractions enriched for nuclei, heavy membranes
- 15 (lysosomes, peroxisomes, and mitochondria), light membranes (plasma membrane and endoplasmic reticulum), and soluble proteins.

#### **Example 11: Identification of Potential Signal Transduction Pathways**

- 20           Many mammalian proteins have been reported to interact with signaling molecules and to participate in regulating signaling pathways. (J Neurochem. 2001; 76:217-223). Using immunoprecipitation and Western blotting techniques, proteins are identified that associate with 55P4H4 and mediate signaling events. Several pathways known to play a role in cancer biology can be regulated by 55P4H4, including phospholipid pathways such as PI3K, AKT, etc.; adhesion and migration pathways, including FAK, Rho, Rac-1, etc.; pathways involved in the regulation of
- 25 transcription such as Wnt (Bioessays. 2001, 23:311) as well as mitogenic/survival cascades such as ERK, p38, STAT, etc (Cell Growth Differ. 2000,11:279; J Biol Chem. 1999, 274:801; Oncogene. 2000, 19:3003; J. Cell Biol. 1997, 138:913.).

- 30           Using Western blotting techniques, the ability of 55P4H4 to regulate of these pathways is examined. Cells expressing or lacking 55P4H4 are either left untreated or stimulated with cytokines, androgen and anti-integrin antibodies. Cell lysates are analyzed using anti-phospho-specific antibodies (Cell Signaling, Santa Cruz Biotechnology) in order to detect phosphorylation and regulation of ERK, p38, AKT, PI3K, PLC and other signaling molecules. When 55P4H4 plays a role in the regulation of signaling pathways are used as a target for diagnostic, prognostic, preventative and therapeutic purposes.



To determine whether 55P4H4 directly or indirectly activates known signal transduction pathways in cells, luciferase (luc) based transcriptional reporter assays are carried out in cells expressing individual genes. These transcriptional reporters contain consensus-binding sites for known transcription factors that lie downstream of well-characterized signal transduction pathways. The reporters and examples of these associated transcription factors, signal transduction pathways, and activation stimuli are listed below.

1. NFkB-luc, NFkB/Rel; Ik-kinase/SAPK; growth/apoptosis/stress
2. SRE-luc, SRF/TCF/ELK1; MAPK/SAPK; growth/differentiation
3. AP-1-luc, FOS/JUN; MAPK/SAPK/PKC; growth/apoptosis/stress
4. ARE-luc, androgen receptor; steroids/MAPK; growth/differentiation/apoptosis
5. p53-luc, p53; SAPK; growth/differentiation/apoptosis
6. CRE-luc, CREB/ATF2; PKA/p38; growth/apoptosis/stress

Gene-mediated effects are assayed in cells showing mRNA expression. Luciferase reporter plasmids can be introduced by lipid-mediated transfection (TFX-50, Promega). Luciferase activity, an indicator of relative transcriptional activity, is measured by incubation of cell extracts with luciferin substrate and luminescence of the reaction is monitored in a luminometer.

Signaling pathways activated by 55P4H4 are mapped and used for the identification and validation of therapeutic targets. When the 55P4H4 gene is involved in cell signaling, it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 12: Involvement in Tumor Progression**

The 55P4H4 gene can contribute to the growth of cancer cells. The role of 55P4H4 in tumor growth is investigated in a variety of primary and transfected cell lines including prostate, colon, bladder and kidney cell lines as well as NIH 3T3 cells engineered to stably express 55P4H4. Parental cells lacking 55P4H4 and cells expressing 55P4H4 are evaluated for cell growth using a well-documented proliferation assay (Fraser SP, Grimes JA, Djamgoz MB. Prostate. 2000;44:61, Johnson DE, Ochieng J, Evans SL. Anticancer Drugs. 1996, 7:288).

To determine the role of 55P4H4 the transformation process, its effect in colony forming assays is evaluated. Parental NIH3T3 cells lacking 55P4H4 are compared to NHI-3T3 cells expressing 55P4H4, using a soft agar assay under stringent and more permissive conditions (Song Z. et al. Cancer Res. 2000;60:6730).

To determine the role of 55P4H4 in invasion and metastasis of cancer cells, a well-established Transwell Insert System assay (Becton Dickinson) (Cancer Res. 1999; 59:6010). Control cells, including prostate, colon, bladder and kidney cell lines lacking 55P4H4 are compared to cells

expressing 55P4H4. Cells are loaded with the fluorescent dye, calcein, and plated in the top well of the Transwell insert coated with a basement membrane analog. Invasion is determined by fluorescence of cells in the lower chamber relative to the fluorescence of the entire cell population.

- 5 55P4H4 can also play a role in cell cycle and apoptosis. Parental cells and cells expressing 55P4H4 are compared for differences in cell cycle regulation using a well-established BrdU assay (Abdel-Malek ZA. J Cell Physiol. 1988, 136:247). In short, cells are grown under both optimal (full serum) and limiting (low serum) conditions are labeled with BrdU and stained with anti-BrdU Ab and propidium iodide. Cells are analyzed for entry into the G1, S, and G2M phases of the cell cycle. Alternatively, the effect of stress on apoptosis is evaluated in control parental cells and cells expressing 55P4H4, including normal and tumor prostate, bladder and lung cells. Engineered and parental cells are treated with various chemotherapeutic agents, such as etoposide, flutamide, etc, and protein synthesis inhibitors, such as cycloheximide. Cells are stained with annexin V-FITC and cell death is measured by FACS analysis. The modulation of cell death by 55P4H4 can play a critical role in regulating tumor progression and tumor load.
- 15 When 55P4H4 plays a role in cell growth, transformation, invasion or apoptosis, it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 13: Regulation of Transcription**

- 20 The potential localization of 55P4H4 to the nucleus and its similarity to HOX-regulated genes indicate that 55P4H4 can play a role in transcriptional regulation of eukaryotic genes. Regulation of gene expression can be evaluated by studying gene expression in cells expressing or lacking 55P4H4. For this purpose, two types of experiments are performed. In the first set of experiments, RNA from parental and 55P4H4-expressing cells are extracted and hybridized to commercially available gene arrays (Clontech) (Smid-Koopman E et al. Br J Cancer. 2000. 83:246). Resting cells as well as cells 25 treated with FBS or androgen are compared. Differentially expressed genes are identified in accordance with procedures known in the art. The differentially expressed genes are then mapped to biological pathways (Chen K et al. Thyroid. 2001. 11:41.).

- 30 In the second set of experiments, specific transcriptional pathway activation is evaluated using commercially available (Stratagene) luciferase reporter constructs including: NFkB-luc, SRE-luc, ELK1-luc, ARE-luc, p53-luc, and CRE-luc. These transcriptional reporters contain consensus binding sites for known transcription factors that lie downstream of well-characterized signal transduction pathways, and represent a good tool to ascertain pathway activation and screen for positive and negative modulators of pathway activation.

- 35 When 55P4H4 plays a role in gene regulation, it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 14: Regulation of 5SP4H4 Expression**

The expression of Hox responsive genes can be regulated by several factors, including hormones, cytokines, growth factors and hypoxia (see, e.g., Ornstein DK et al. J Urol. 2001, 165:1329; Lin B et al, Cancer Res. 2000, 60:858; Kumar JP and Moses K, Cell 2001, 104:687). Using xenograft cells as well as other 5SP4H4 expressing cells, the regulation of 5SP4H4 expression by hormones, such as androgen, estrogen, glucocorticoids, retinoic acid and progesterone is studied. Similarly, the effect of hypoxia, growth factors, secreted proteins and small molecules on 5SP4H4 expression is investigated in cells treated with EGF, FGF, VEGF, TGF, IGF and other proteins. These studies identify small molecules that regulate the expression of 5SP4H4, and are valuable in therapeutic molecules. When the expression of 5SP4H4 is modified by proteins and small molecules, it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 15: Identification of Hox-Response Element in the 5SP4H4-Promoter**

Hox proteins bind to DNA in a sequence specific manner, thereby regulating gene expression in genes carrying the homeobox recognition sequence (Norris P et al, DNA Cell Biol. 2001, 20:89; Sur IP and Toftgard R. Mol Cell Biol Res Commun. 2000, 3:367). Using electrophoretic mobility shift assays (EMSA) and DNA footprinting, HOX-binding response elements are identified in the 5SP4H4 promoter sequence. In short, nuclear lysates are extracted from parental 5SP4H4-negative as well as 5SP4H4-expressing cells. The lysates are incubated in the presence of <sup>32</sup>P-labeled DNA probes representing various segments of the 5SP4H4 promoter region. DNA-protein complexes are either separated by electrophoresis or exposed to a restriction nuclease, and analyzed by radiography. Proteins that bind to the 5SP4H4 promoter are identified using Hox specific antibodies. To confirm Hox binding to 5SP4H4 upstream elements, <sup>32</sup>P-labeled 5SP4H4 probes are incubated in the presence or absence of various Hox proteins and analyzed as above.

In another embodiment, the 5SP4H4 promoter is linked to a reporter construct, such as luciferase or  $\beta$ -galactocidase. The 5SP4H4 reporter construct is introduced into 5SP4H4-negative as well as 5SP4H4-expressing cells, and reporter activity is evaluated using commercially available detection methods. These experiments identify elements that regulate 5SP4H4 expression and therefore the effect of 5SP4H4 and thereby are valuable tools in designing and testing inhibitors of 5SP4H4. When 5SP4H4 is regulated by Hox transcription factors, these are used for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 16: Involvement of 5SP4H4 in Protein Trafficking.**

Due to its similarity to Ric-1, 5SP4H4 may regulate intracellular trafficking (Bensen ES, Yeung BG, Payne GS. Mol Biol Cell. 2001, 12:13). Trafficking of proteins is studied using well-established methods (see, e.g., Valetti C. et al. Mol Biol Cell. 1999, 10:4107). In short, FITC-

conjugated  $\alpha$ 2-macroglobulin is incubated with 55P4H4-expressing and 55P4H4-negative cells. The location and uptake of FITC- $\alpha$ 2-macroglobulin are visualized using a fluorescent microscope.

In another set of experiments, the co-localization of 55P4H4 with golgi-associated proteins is evaluated by co-precipitation and Western blotting techniques as well as fluorescent microscopy. Briefly, cells are allowed to inject the labeled BSA and are placed intermittently at 4°C and 18°C to allow for trafficking to take place. Cells are examined under fluorescent microscopy at different time points for the presence of labeled BSA in specific vesicular compartments, including Golgi, endoplasmic reticulum, etc. Using such assay systems, proteins, antibodies and small molecules are identified that modify the effect of 55P4H4 on vesicular transport. When 55P4H4 plays a role in intracellular trafficking, 55P4H4 is a target for diagnostic, preventative and therapeutic purposes

#### **Example 17: Protein-Protein Association**

Based on its homology to Ric-1, 55P4H4 can regulate cell division, gene transcription, and cell transformation by associating with other molecules (Siniossoglou S, Peak-Chew SY, Pelham HR EMBO J. 2000, 19:4885). Using immunoprecipitation techniques as well as two yeast hybrid systems, proteins are identified that associate with 55P4H4. Immunoprecipitates from cells expressing 55P4H4 and cells lacking 55P4H4 are compared for specific protein-protein associations.

55P4H4 may also associate with signaling molecules such as Smads (J Biol Chem. 2000, 275:8267), effector molecules such as adaptor proteins and SH2-containing proteins. Studies comparing 55P4H4 positive and 55P4H4 negative cells as well as studies comparing unstimulated/resting cells and cells treated with epithelial cell activators, such as cytokines, growth factors, androgen and anti-integrin antibodies reveal unique interactions. In addition, protein-protein interactions are studied using two yeast hybrid methodology (Curr Opin Chem Biol. 1999, 3:64). A vector carrying a library of proteins fused to the activation domain of a transcription factor is introduced into yeast expressing a 55P4H4-DNA-binding domain fusion protein and a reporter construct. Protein-protein interactions are detected by calorimetric reporter activity. Specific association with effector molecules and transcription factors directs one of skill to the mode of action of 55P4H4, and thus identifies therapeutic, prognostic, preventative and/or diagnostic targets for cancer. This and similar assays are also be used to identify and screen for small molecules that interact with 55P4H4. When 55P4H4 associates with proteins or small molecules it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 18: Involvement in Angiogenesis**

Angiogenesis or new capillary blood vessel formation is necessary for tumor growth (Hanahan D, Folkman J. Cell. 1996, 86:353; Folkman J. Endocrinology. 1998 139:441). Several assays have been developed to measure angiogenesis *in vitro* and *in vivo*, for example tissue culture assays that

evaluate endothelial cell tube formation and endothelial cell proliferation. Using these assays as well as *in vitro* neo-vascularization, one determines whether 55P4H4 enhances or inhibits angiogenesis.

For example, endothelial cells and cell lines are plated on an artificial basement membrane, such as matrigel, in the presence and absence of 55P4H4. The effect on tube formation is evaluated using light microscopy. In another embodiment, endothelial cells engineered to express 55P4H4 are evaluated using tube formation and proliferation assays. The effect of 55P4H4 is also evaluated in animal models *in vivo*. For example, cells either expressing or lacking 55P4H4 are implanted subcutaneously in immunocompromised mice. Endothelial cell migration and angiogenesis are evaluated 5-15 days later using immunohistochemistry techniques. When 55P4H4 affects angiogenesis, it is used as a target for diagnostic, prognostic, preventative and therapeutic purposes.

#### **Example 19: 55P4H4 Monoclonal Antibody-mediated Inhibition of Prostate Tumors *In Vivo***

The significant expression of 55P4H4, in cancer tissues, together with its restrictive expression in normal tissues makes 55P4H4 an excellent target for antibody therapy. Similarly, 55P4H4 is a target for T cell-based immunotherapy. Thus, the therapeutic efficacy of anti-55P4H4 mAbs in human prostate cancer xenograft mouse models is evaluated by using androgen-independent LAPC-4 and LAPC-9 xenografts (Craft, N., *et al.*, Cancer Res, 1999, 59(19): p. 5030-6) and the androgen independent recombinant cell line PC3-55P4H4 (see, e.g., Kaighn, M.E., *et al.*, Invest Urol, 1979, 17(1): p. 16-23).

Antibody efficacy on tumor growth and metastasis formation is studied, e.g., in a mouse orthotopic prostate cancer xenograft model. The antibodies can be unconjugated, as discussed in this Example, or can be conjugated to a therapeutic modality, as appreciated in the art. We demonstrate that anti-55P4H4 mAbs inhibit formation of both the androgen-dependent LAPC-9 and androgen-independent PC3-55P4H4 tumor xenografts. Anti-55P4H4 mAbs also retard the growth of established orthotopic tumors and prolonged survival of tumor-bearing mice. These results indicate the utility of anti-55P4H4 mAbs in the treatment of local and advanced stages of prostate cancer. (See, e.g., (Saffran, D., *et al.*, PNAS 10:1073-1078 or [www.pnas.org/cgi/doi/10.1073/pnas.051624698](http://www.pnas.org/cgi/doi/10.1073/pnas.051624698))

Administration of the anti-55P4H4 mAbs led to retardation of established orthotopic tumor growth and inhibition of metastasis to distant sites, resulting in a significant prolongation in the survival of tumor-bearing mice. These studies indicate that 55P4H4 as an attractive target for immunotherapy and demonstrate the therapeutic potential of anti-55P4H4 mAbs for the treatment of local and metastatic prostate cancer. This example demonstrates that unconjugated 55P4H4 monoclonal antibodies are effective to inhibit the growth of human prostate tumor xenografts grown in SCID mice; accordingly a combination of such efficacious monoclonal antibodies is also effective.

## Tumor inhibition using multiple unconjugated 55P4H4 mAbs

### Materials and Methods

#### 55P4H4 Monoclonal Antibodies:

5 Monoclonal antibodies are raised against 55P4H4 as described in Example 7. The antibodies are characterized by ELISA, Western blot, FACS, and immunoprecipitation for their capacity to bind 55P4H4. Epitope mapping data for the anti-55P4H4 mAbs, as determined by ELISA and Western analysis, recognize epitopes on the 55P4H4 protein. Immunohistochemical analysis of prostate cancer tissues and cells with these antibodies is performed.

10 The monoclonal antibodies are purified from ascites or hybridoma tissue culture supernatants by Protein-G Sepharose chromatography, dialyzed against PBS, filter sterilized, and stored at -20°C. Protein determinations are performed by a Bradford assay (Bio-Rad, Hercules, CA). A therapeutic monoclonal antibody or a cocktail comprising a mixture of individual monoclonal antibodies is prepared and used for the treatment of mice receiving subcutaneous or orthotopic injections of LAPC-9 prostate tumor xenografts.

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#### Prostate Cancer Xenografts and Cell Lines.

20 The LAPC-9 xenograft, which expresses a wild-type androgen receptor and produces prostate-specific antigen (PSA), is passaged in 6- to 8-week-old male ICR-severe combined immunodeficient (SCID) mice (Taconic Farms) by s.c. trocar implant (Craft, N., *et al.*, supra). Single-cell suspensions of LAPC-9 tumor cells are prepared as described in Craft, *et al.* The prostate carcinoma cell line PC3 (American Type Culture Collection) is maintained in DMEM supplemented with L-glutamine and 10% (vol/vol) FBS.

25 A PC3-55P4H4 cell population is generated by retroviral gene transfer as described in Hubert, R.S., *et al.*, *STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors*. Proc Natl Acad Sci U S A, 1999. 96(25): p. 14523-8. Anti-55P4H4 staining is detected by using an FITC-conjugated goat anti-mouse antibody (Southern Biotechnology Associates) followed by analysis on a Coulter Epics-XL flow cytometer.

#### Xenograft Mouse Models.

30 Subcutaneous (s.c.) tumors are generated by injection of  $1 \times 10^6$  LAPC-9, PC3, or PC3-55P4H4 cells mixed at a 1:1 dilution with Matrigel (Collaborative Research) in the right flank of male SCID mice. To test antibody efficacy on tumor formation, i.p. antibody injections are started on the same day as tumor-cell injections. As a control, mice are injected with either purified mouse IgG (ICN) or PBS; or a purified monoclonal antibody that recognizes an irrelevant antigen not expressed in human cells. In preliminary studies, no difference is found between mouse IgG or PBS on tumor growth. Tumor sizes are determined by vernier caliper measurements, and the tumor volume is

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calculated as length x width x height. Mice with s.c. tumors greater than 1.5 cm in diameter are sacrificed. PSA levels are determined by using a PSA ELISA kit (Anogen, Mississauga, Ontario). Circulating levels of anti-55P4H4 mAbs are determined by a capture ELISA kit (Bethyl Laboratories, Montgomery, TX). (See, e.g., (Saffran, D., et al., PNAS 10:1073-1078 or [www.pnas.org/cgi/doi/10.1073/pnas.051624698](http://www.pnas.org/cgi/doi/10.1073/pnas.051624698))

Orthotopic injections are performed under anesthesia by using ketamine/xylazine. An incision is made through the abdominal muscles to expose the bladder and seminal vesicles, which then are delivered through the incision to expose the dorsal prostate. LAPC-9 cells ( $5 \times 10^5$ ) mixed with Matrigel are injected into each dorsal lobe in a 10- $\mu$ l volume. To monitor tumor growth, mice are bled on a weekly basis for determination of PSA levels. Based on the PSA levels, the mice are segregated into groups for the appropriate treatments. To test the effect of anti-55P4H4 mAbs on established orthotopic tumors, i.p. antibody injections are started when PSA levels reach 2–80 ng/ml.

#### Anti-55P4H4 mAbs Inhibit Growth of 55P4H4-Expressing Prostate-Cancer Tumors

We next test the effect of anti-55P4H4 mAbs on tumor formation by using the LAPC-9 orthotopic model. As compared with the s.c. tumor model, the orthotopic model, which requires injection of tumor cells directly in the mouse prostate, results in a local tumor growth, development of metastasis in distal sites, deterioration of mouse health, and subsequent death (Saffran, D., et al., PNAS supra; Fu, X., et al., Int J Cancer, 1992. 52(6): p. 987-90; Kubota, T., J Cell Biochem, 1994. 56(1): p. 4-8). The features make the orthotopic model more representative of human disease progression and allowed us to follow the therapeutic effect of mAbs on clinically relevant end points.

Accordingly, LAPC-9 tumor cells are injected into the mouse prostate, and 2 days later, the mice are segregated into two groups and treated with either up to 200 $\mu$ g, usually 10-50 $\mu$ g, of anti-55P4H4 Ab or PBS three times per week for two to five weeks. Mice are monitored weekly for circulating PSA levels as an indicator of tumor growth.

A major advantage of the orthotopic prostate-cancer model is the ability to study the development of metastases. Formation of metastasis in mice bearing established orthotopic tumors is studied by IHC analysis on lung sections using an antibody against a prostate-specific cell-surface protein STEAP expressed at high levels in LAPC-9 xenografts (Hubert, R.S., et al., Proc Natl Acad Sci U S A, 1999. 96(25): p. 14523-8).

Mice bearing established orthotopic LAPC-9 tumors are administered 11 injections of either anti-55P4H4 mAb or PBS over a 4-week period. Mice in both groups are allowed to establish a high tumor burden (PSA levels greater than 300 ng/ml), to ensure a high frequency of metastasis formation in mouse lungs. Mice then are killed and their prostate and lungs are analyzed for the presence of LAPC-9 cells by anti-STEAP IHC analysis.

These studies demonstrate a broad anti-tumor efficacy of anti-55P4H4 antibodies on initiation and progression of prostate cancer in xenograft mouse models. Anti-55P4H4 antibodies inhibit tumor formation of both androgen-dependent and androgen-independent tumors as well as retarding the growth of already established tumors and prolong the survival of treated mice. Moreover, anti-55P4H4 mAbs demonstrate a dramatic inhibitory effect on the spread of local prostate tumor to distal sites, even in the presence of a large tumor burden. Thus, anti-55P4H4 mAbs are efficacious on major clinically relevant end points/PSA levels (tumor growth), prolongation of survival, and health.

#### **Example 20: Androgen Regulation of 55P4H4**

To determine if 55P4H4 is regulated by androgen, LAPC-4 AD and LAPC-9 AD cells are grown in charcoal-stripped medium and stimulated with the synthetic androgen mibolerone, for either 14 or 24 hours. Expression of 55P4H4 is studied before and after stimulation with mibolerone. The experimental samples are confirmed by testing for the expression of the androgen-regulated prostate cancer gene PSA. In another experiment, 55P4H4 expression is analyzed in LAPC-9 AD and LAPC-9 AI tumors grown in castrated mice. Only, androgen independent tumors will grow in castrated mice.

When 55P4H4 expression is regulated by androgen, 55P4H4 is a target for diagnostic, preventative and therapeutic purposes.

#### **Example 21: Tissue-targeted Therapeutic Strategies**

Given the expression of 55P4H4 in select normal tissues and in various cancer tissues, strategies can be employed to direct therapeutic 55P4H4 molecules to specific target tissues, as appropriate to an individual therapeutic application. For example, therapeutic compositions containing 55P4H4-related molecules are used for intravesical administration to treat kidney or bladder cancer. To treat prostate cancer, in another example, a therapeutic composition is injected within the prostatic capsule or directly within the tumor. In yet another example, aerosol administration is used to target lung tissue. Therapeutic compositions containing 55P4H4-related molecules are used for intratumoral administration to treat testicular, brain or bone cancer. Therapeutic compositions containing 55P4H4-related molecules are used for intraluminal or intratumoral administration to treat cervical cancer. Therapeutic compositions containing 55P4H4-related molecules are used for intratumoral administration or injection into the ovarian capsule to treat ovarian cancer. Optionally any of the above modalities comprise pharmaceutical excipients to minimize systemic adsorption. Other methods of targeting therapeutic compositions to specific tissues are known in the art.



#### **Example 22: HLA Class I and Class II Binding Assays**

HLA class I and class II binding assays using purified HLA molecules are performed in accordance with disclosed protocols (e.g., PCT publications WO 94/20127 and WO 94/03205; Sidney *et al.*, *Current Protocols in Immunology* 18.3.1 (1998); Sidney, *et al.*, *J. Immunol.* 154:247 (1995); Sette, *et al.*, *Mol. Immunol.* 31:813 (1994)). Briefly, purified MHC molecules (5 to 500nM) are incubated with various unlabeled peptide inhibitors and 1-10nM <sup>125</sup>I-radiolabeled probe peptides as described. Following incubation, MHC-peptide complexes are separated from free peptide by gel filtration and the fraction of peptide bound is determined. Typically, in preliminary experiments, each MHC preparation is titrated in the presence of fixed amounts of radiolabeled peptides to determine the concentration of HLA molecules necessary to bind 10-20% of the total radioactivity. All subsequent inhibition and direct binding assays are performed using these HLA concentrations.

Since under these conditions [label]<[HLA] and IC<sub>50</sub>≥[HLA], the measured IC<sub>50</sub> values are reasonable approximations of the true K<sub>D</sub> values. Peptide inhibitors are typically tested at concentrations ranging from 120 µg/ml to 1.2 ng/ml, and are tested in two to four completely independent experiments. To allow comparison of the data obtained in different experiments, a relative binding figure is calculated for each peptide by dividing the IC<sub>50</sub> of a positive control for inhibition by the IC<sub>50</sub> for each tested peptide (typically unlabeled versions of the radiolabeled probe peptide). For database purposes, and inter-experiment comparisons, relative binding values are compiled. These values can subsequently be converted back into IC<sub>50</sub> nM values by dividing the IC<sub>50</sub> nM of the positive controls for inhibition by the relative binding of the peptide of interest. This method of data compilations accurate and consistent for comparing peptides that have been tested on different days, or with different lots of purified MHC.

Binding assays as outlined above may be used to analyze HLA supermotif and/or HLA motif-bearing peptides.

#### **Example 23: Identification of HLA Supermotif- and Motif-Bearing CTL Candidate Epitopes**

HLA vaccine compositions of the invention can include multiple epitopes. The multiple epitopes can comprise multiple HLA supermotifs or motifs to achieve broad population coverage. This example illustrates the identification of supermotif- and motif-bearing epitopes for the inclusion in such a vaccine composition. Calculation of population coverage is performed using the strategy described below.

Computer searches and algorithms for identification of supermotif and/or motif-bearing epitopes

The searches performed to identify the motif-bearing peptide sequences disclosed herein employ the protein sequence data from the gene product of 55P4H4 set forth in Figures 2 and 3.

5 Computer searches for epitopes bearing HLA Class I or Class II supermotifs or motifs are performed as follows. All translated 55P4H4 protein sequences are analyzed using a text string search software program to identify potential peptide sequences containing appropriate HLA binding motifs; such programs are readily produced in accordance with information in the art in view of known motif/supermotif disclosures. Furthermore, such calculations can be made mentally.

10 Identified A2-, A3-, and DR-supermotif sequences are scored using polynomial algorithms to predict their capacity to bind to specific HLA-Class I or Class II molecules. These polynomial algorithms account for the impact of different amino acids at different positions, and are essentially based on the premise that the overall affinity (or  $\Delta G$ ) of peptide-HLA molecule interactions can be approximated as a linear polynomial function of the type:

15 
$$"\Delta G" = a_{1i} \times a_{2i} \times a_{3i} \dots \times a_{ni}$$

where  $a_{ji}$  is a coefficient which represents the effect of the presence of a given amino acid ( $j$ ) at a given position ( $i$ ) along the sequence of a peptide of  $n$  amino acids. The crucial assumption of this method is that the effects at each position are essentially independent of each other (i.e., independent binding of individual side-chains). When residue  $j$  occurs at position  $i$  in the peptide, it is assumed to contribute a constant amount  $j_i$  to the free energy of binding of the peptide irrespective of the sequence of the rest of the peptide.

20 The method of derivation of specific algorithm coefficients has been described in Gulukota *et al.*, *J. Mol. Biol.* 267:1258-126, 1997; (see also Sidney *et al.*, *Human Immunol.* 45:79-93, 1996; and Southwood *et al.*, *J. Immunol.* 160:3363-3373, 1998). Briefly, for all  $i$  positions, anchor and non-anchor alike, the geometric mean of the average relative binding (ARB) of all peptides carrying  $j$  is calculated relative to the remainder of the group, and used as the estimate of  $j_i$ . For Class II peptides, if multiple alignments are possible, only the highest scoring alignment is utilized, following an iterative procedure. To calculate an algorithm score of a given peptide in a test set, the ARB values corresponding to the sequence of the peptide are multiplied. If this product exceeds a chosen threshold, the peptide is predicted to bind. Appropriate thresholds are chosen as a function of the degree of stringency of prediction desired.

Selection of HLA-A2 supertype cross-reactive peptides

25 Complete protein sequences from 55P4H4 are scanned utilizing motif identification software, to identify 8-, 9- 10- and 11-mer sequences containing the HLA-A2-supermotif main anchor specificity. Typically, these sequences are then scored using the protocol described above and the peptides corresponding to the positive-scoring sequences are synthesized and tested for their capacity

to bind purified HLA-A\*0201 molecules *in vitro* (HLA-A\*0201 is considered a prototype A2 supertype molecule).

5 These peptides are then tested for the capacity to bind to additional A2-supertype molecules (A\*0202, A\*0203, A\*0206, and A\*6802). Peptides that bind to at least three of the five A2-supertype alleles tested are typically deemed A2-supertype cross-reactive binders. Preferred peptides bind at an affinity equal to or less than 500 nM to three or more HLA-A2 supertype molecules.

#### Selection of HLA-A3 supermotif-bearing epitopes

10 The 55P4H4 protein sequence scanned above is also examined for the presence of peptides with the HLA-A3-supermotif primary anchors. Peptides corresponding to the HLA A3 supermotif-bearing sequences are then synthesized and tested for binding to HLA-A\*0301 and HLA-A\*1101 molecules, the molecules encoded by the two most prevalent A3-supertype alleles. The peptides that bind at least one of the two alleles with binding affinities of  $\leq 500$  nM, often  $\leq 200$  nM, are then tested for binding cross-reactivity to the other common A3-supertype alleles (e.g., A\*3101, A\*3301, and A\*6801) to identify those that can bind at least three of the five HLA-A3-supertype molecules tested.

#### 15 Selection of HLA-B7 supermotif bearing epitopes

The 55P4H4 protein is also analyzed for the presence of 8-, 9-, 10-, or 11-mer peptides with the HLA-B7-supermotif. Corresponding peptides are synthesized and tested for binding to HLA-B\*0702, the molecule encoded by the most common B7-supertype allele (*i.e.*, the prototype B7 supertype allele). Peptides binding B\*0702 with  $IC_{50}$  of  $\leq 500$  nM are identified using standard methods. These peptides are then tested for binding to other common B7-supertype molecules (e.g., B\*3501, B\*5101, B\*5301, and B\*5401). Peptides capable of binding to three or more of the five B7-supertype alleles tested are thereby identified.

#### Selection of A1 and A24 motif-bearing epitopes

25 To further increase population coverage, HLA-A1 and -A24 epitopes can also be incorporated into vaccine compositions. An analysis of the 55P4H4 protein can also be performed to identify HLA-A1- and A24-motif-containing sequences.

High affinity and/or cross-reactive binding epitopes that bear other motif and/or supermotifs are identified using analogous methodology.

#### 30 Example 24: Confirmation of Immunogenicity

Cross-reactive candidate CTL A2-supermotif-bearing peptides that are identified as described herein are selected for *in vitro* immunogenicity testing. Testing is performed using the following methodology:

#### Target Cell Lines for Cellular Screening:

The .221A2.1 cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human B-lymphoblastoid cell line 721.221, is used as the peptide-loaded target to measure activity of HLA-A2.1-restricted CTL. This cell line is grown in RPMI-1640 medium supplemented with antibiotics, sodium pyruvate, nonessential amino acids and 10% (v/v) heat inactivated FCS. Cells that express an antigen of interest, or transfectants comprising the gene encoding the antigen of interest, can be used as target cells to test the ability of peptide-specific CTLs to recognize endogenous antigen.

Primary CTL Induction Cultures:

*Generation of Dendritic Cells (DC):* PBMCs are thawed in RPMI with 30 µg/ml DNase, washed twice and resuspended in complete medium (RPMI-1640 plus 5% AB human serum, non-essential amino acids, sodium pyruvate, L-glutamine and penicillin/streptomycin). The monocytes are purified by plating  $10 \times 10^6$  PBMC/well in a 6-well plate. After 2 hours at 37°C, the non-adherent cells are removed by gently shaking the plates and aspirating the supernatants. The wells are washed a total of three times with 3 ml RPMI to remove most of the non-adherent and loosely adherent cells. Three ml of complete medium containing 50 ng/ml of GM-CSF and 1,000 U/ml of IL-4 are then added to each well. TNFα is added to the DCs on day 6 at 75 ng/ml and the cells are used for CTL induction cultures on day 7.

*Induction of CTL with DC and Peptide:* CD8+ T-cells are isolated by positive selection with Dynal immunomagnetic beads (Dynabeads® M-450) and the detach-a-bead® reagent. Typically about  $200\text{--}250 \times 10^6$  PBMC are processed to obtain  $24 \times 10^6$  CD8+ T-cells (enough for a 48-well plate culture). Briefly, the PBMCs are thawed in RPMI with 30 µg/ml DNase, washed once with PBS containing 1% human AB serum and resuspended in PBS/1% AB serum at a concentration of  $20 \times 10^6$  cells/ml. The magnetic beads are washed 3 times with PBS/AB serum, added to the cells (140 µl beads/ $20 \times 10^6$  cells) and incubated for 1 hour at 4°C with continuous mixing. The beads and cells are washed 4x with PBS/AB serum to remove the nonadherent cells and resuspended at  $100 \times 10^6$  cells/ml (based on the original cell number) in PBS/AB serum containing 100 µl/ml detach-a-bead® reagent and 30 µg/ml DNase. The mixture is incubated for 1 hour at room temperature with continuous mixing. The beads are washed again with PBS/AB/DNase to collect the CD8+ T-cells. The DC are collected and centrifuged at 1300 rpm for 5-7 minutes, washed once with PBS with 1% BSA, counted and pulsed with 40 µg/ml of peptide at a cell concentration of  $1\text{--}2 \times 10^6$ /ml in the presence of 3 µg/ml β<sub>2</sub>-microglobulin for 4 hours at 20°C. The DC are then irradiated (4,200 rads), washed 1 time with medium and counted again.

*Setting up induction cultures:* 0.25 ml cytokine-generated DC ( $@1 \times 10^5$  cells/ml) are co-cultured with 0.25 ml of CD8+ T-cells ( $@2 \times 10^6$  cell/ml) in each well of a 48-well plate in the presence of 10 ng/ml of IL-7. Recombinant human IL-10 is added the next day at a final concentration of 10 ng/ml and rhuman IL-2 is added 48 hours later at 10 IU/ml.

Restimulation of the induction cultures with peptide-pulsed adherent cells: Seven and fourteen days after the primary induction, the cells are restimulated with peptide-pulsed adherent cells. The PBMCs are thawed and washed twice with RPMI and DNase. The cells are resuspended at  $5 \times 10^6$  cells/ml and irradiated at ~4200 rads. The PBMCs are plated at  $2 \times 10^6$  in 0.5ml complete medium per well and incubated for 2 hours at 37°C. The plates are washed twice with RPMI by tapping the plate gently to remove the nonadherent cells and the adherent cells pulsed with 10µg/ml of peptide in the presence of 3 µg/ml  $\beta_2$  microglobulin in 0.25ml RPMI/5%AB per well for 2 hours at 37°C. Peptide solution from each well is aspirated and the wells are washed once with RPMI. Most of the media is aspirated from the induction cultures (CD8+ cells) and brought to 0.5 ml with fresh media. The cells are then transferred to the wells containing the peptide-pulsed adherent cells. Twenty four hours later recombinant human IL-10 is added at a final concentration of 10ng/ml and recombinant human IL2 is added the next day and again 2-3 days later at 50IU/ml (Tsai *et al.*, *Critical Reviews in Immunology* 18(1-2):65-75, 1998). Seven days later, the cultures are assayed for CTL activity in a  $^{51}\text{Cr}$  release assay. In some experiments the cultures are assayed for peptide-specific recognition in the *in situ* IFN $\gamma$  ELISA at the time of the second restimulation followed by assay of endogenous recognition 7 days later. After expansion, activity is measured in both assays for a side-by-side comparison.

Measurement of CTL lytic activity by  $^{51}\text{Cr}$  release.

Seven days after the second restimulation, cytotoxicity is determined in a standard (5hr)  $^{51}\text{Cr}$  release assay by assaying individual wells at a single E:T. Peptide-pulsed targets are prepared by incubating the cells with 10µg/ml peptide overnight at 37°C.

Adherent target cells are removed from culture flasks with trypsin-EDTA. Target cells are labeled with 200µCi of  $^{51}\text{Cr}$  sodium chromate (Dupont, Wilmington, DE) for 1 hour at 37°C. Labeled target cells are resuspended at  $10^6$  per ml and diluted 1:10 with K562 cells at a concentration of  $3.3 \times 10^6$ /ml (an NK-sensitive erythroblastoma cell line used to reduce non-specific lysis). Target cells (100 µl) and effectors (100µl) are plated in 96 well round-bottom plates and incubated for 5 hours at 37°C. At that time, 100 µl of supernatant are collected from each well and percent lysis is determined according to the formula:

$$[(\text{cpm of the test sample} - \text{cpm of the spontaneous } ^{51}\text{Cr release sample}) / (\text{cpm of the maximal } ^{51}\text{Cr release sample} - \text{cpm of the spontaneous } ^{51}\text{Cr release sample})] \times 100.$$

Maximum and spontaneous release are determined by incubating the labeled targets with 1% Triton X-100 and media alone, respectively. A positive culture is defined as one in which the specific lysis (sample- background) is 10% or higher in the case of individual wells and is 15% or more at the two highest E:T ratios when expanded cultures are assayed.

*In situ* Measurement of Human IFN $\gamma$  Production as an Indicator of Peptide-specific and Endogenous Recognition

Immulon 2 plates are coated with mouse anti-human IFN $\gamma$  monoclonal antibody (4  $\mu$ g/ml 0.1M NaHCO $_3$ , pH8.2) overnight at 4°C. The plates are washed with Ca $^{2+}$ , Mg $^{2+}$ -free PBS/0.05% Tween 20 and blocked with PBS/10% FCS for two hours, after which the CTLs (100  $\mu$ l/well) and targets (100  $\mu$ l/well) are added to each well, leaving empty wells for the standards and blanks (which received media only). The target cells, either peptide-pulsed or endogenous targets, are used at a concentration of 1x10 $^6$  cells/ml. The plates are incubated for 48 hours at 37°C with 5% CO $_2$ .

Recombinant human IFN $\gamma$  is added to the standard wells starting at 400 pg or 1200pg/100 $\mu$ l/well and the plate incubated for two hours at 37°C. The plates are washed and 100  $\mu$ l of biotinylated mouse anti-human IFN $\gamma$  monoclonal antibody (2 $\mu$ g/ml in PBS/3%FCS/0.05% Tween 20) are added and incubated for 2 hours at room temperature. After washing again, 100  $\mu$ l HRP-streptavidin (1:4000) are added and the plates incubated for one hour at room temperature. The plates are then washed 6x with wash buffer, 100 $\mu$ l/well developing solution (TMB 1:1) are added, and the plates allowed to develop for 5-15 minutes. The reaction is stopped with 50  $\mu$ l/well 1M H $_3$ PO $_4$  and read at OD450. A culture is considered positive if it measured at least 50 pg of IFN $\gamma$ /well above background and is twice the background level of expression.

#### CTL Expansion.

Those cultures that demonstrate specific lytic activity against peptide-pulsed targets and/or tumor targets are expanded over a two week period with anti-CD3. Briefly, 5x10 $^4$  CD8+ cells are added to a T25 flask containing the following: 1x10 $^6$  irradiated (4,200 rad) PBMC (autologous or allogeneic) per ml, 2x10 $^5$  irradiated (8,000 rad) EBV- transformed cells per ml, and OKT3 (anti-CD3) at 30ng per ml in RPMI-1640 containing 10% (v/v) human AB serum, non-essential amino acids, sodium pyruvate, 25 $\mu$ M 2-mercaptoethanol, L-glutamine and penicillin/streptomycin. Recombinant human IL2 is added 24 hours later at a final concentration of 200IU/ml and every three days thereafter with fresh media at 50IU/ml. The cells are split if the cell concentration exceeds 1x10 $^6$ /ml and the cultures are assayed between days 13 and 15 at E:T ratios of 30, 10, 3 and 1:1 in the  $^{51}$ Cr release assay or at 1x10 $^6$ /ml in the *in situ* IFN $\gamma$  assay using the same targets as before the expansion.

Cultures are expanded in the absence of anti-CD3 $^+$  as follows. Those cultures that demonstrate specific lytic activity against peptide and endogenous targets are selected and 5x10 $^4$  CD8 $^+$  cells are added to a T25 flask containing the following: 1x10 $^6$  autologous PBMC per ml which have been peptide-pulsed with 10  $\mu$ g/ml peptide for two hours at 37°C and irradiated (4,200 rad); 2x10 $^5$  irradiated (8,000 rad) EBV-transformed cells per ml RPMI-1640 containing 10%(v/v) human AB serum, non-essential AA, sodium pyruvate, 25mM 2-ME, L-glutamine and gentamicin.

#### Immunogenicity of A2 supermotif-bearing peptides

A2-supermotif cross-reactive binding peptides are tested in the cellular assay for the ability to induce peptide-specific CTL in normal individuals. In this analysis, a peptide is typically considered to

be an epitope if it induces peptide-specific CTLs in at least individuals, and preferably, also recognizes the endogenously expressed peptide.

Immunogenicity can also be confirmed using PBMCs isolated from patients bearing a tumor that expresses 55P4H4. Briefly, PBMCs are isolated from patients, re-stimulated with peptide-pulsed monocytes and assayed for the ability to recognize peptide-pulsed target cells as well as transfected cells endogenously expressing the antigen.

#### Evaluation of A\*03/A11 immunogenicity

HLA-A3 supermotif-bearing cross-reactive binding peptides are also evaluated for immunogenicity using methodology analogous for that used to evaluate the immunogenicity of the HLA-A2 supermotif peptides.

#### Evaluation of B7 immunogenicity

Immunogenicity screening of the B7-supertype cross-reactive binding peptides identified as set forth herein are evaluated in a manner analogous to the evaluation of A2-and A3-supermotif-bearing peptides.

Peptides bearing other supermotifs/motifs, *e.g.*, HLA-A1, HLA-A24 *etc.* are also evaluated using similar methodology

#### **Example 25: Implementation of the Extended Supermotif to Improve the Binding Capacity of Native Epitopes by Creating Analogs**

HLA motifs and supermotifs (comprising primary and/or secondary residues) are useful in the identification and preparation of highly cross-reactive native peptides, as demonstrated herein. Moreover, the definition of HLA motifs and supermotifs also allows one to engineer highly cross-reactive epitopes by identifying residues within a native peptide sequence which can be analoged to confer upon the peptide certain characteristics, *e.g.* greater cross-reactivity within the group of HLA molecules that comprise a supertype, and/or greater binding affinity for some or all of those HLA molecules. Examples of analoging peptides to exhibit modulated binding affinity are set forth in this example.

#### Analoging at Primary Anchor Residues

Peptide engineering strategies are implemented to further increase the cross-reactivity of the epitopes. For example, the main anchors of A2-supermotif-bearing peptides are altered, for example, to introduce a preferred L, I, V, or M at position 2, and I or V at the C-terminus.

To analyze the cross-reactivity of the analog peptides, each engineered analog is initially tested for binding to the prototype A2 supertype allele A\*0201, then, if A\*0201 binding capacity is maintained, for A2-supertype cross-reactivity.

Alternatively, a peptide is tested for binding to one or all supertype members and then analogued to modulate binding affinity to any one (or more) of the supertype members to add population coverage.

5 The selection of analogs for immunogenicity in a cellular screening analysis is typically further restricted by the capacity of the parent wild type (WT) peptide to bind at least weakly, i.e., bind at an IC50 of 5000nM or less, to three or more A2 supertype alleles. The rationale for this requirement is that the WT peptides must be present endogenously in sufficient quantity to be biologically relevant. Analogued peptides have been shown to have increased immunogenicity and cross-reactivity by T cells specific for the parent epitope (*see, e.g., Parkhurst et al., J. Immunol. 157:2539, 1996; and Pogue et al.,*  
10 *Proc. Natl. Acad. Sci. USA 92:8166, 1995*).

In the cellular screening of these peptide analogs, it is important to demonstrate that analog-specific CTLs are also able to recognize the wild-type peptide and, when possible, target cells that endogenously express the epitope.

#### Analoging of HLA-A3 and B7-supermotif-bearing peptides

15 Analogs of HLA-A3 supermotif-bearing epitopes are generated using strategies similar to those employed in analoging HLA-A2 supermotif-bearing peptides. For example, peptides binding to 3/5 of the A3-supertype molecules are engineered at primary anchor residues to possess a preferred residue (V, S, M, or A) at position 2.

The analog peptides are then tested for the ability to bind A\*03 and A\*11 (prototype A3  
20 supertype alleles). Those peptides that demonstrate  $\leq 500$  nM binding capacity are then tested for A3-supertype cross-reactivity.

Similarly to the A2- and A3- motif bearing peptides, peptides binding 3 or more B7-supertype alleles can be improved, where possible, to achieve increased cross-reactive binding or greater binding affinity or binding half life. B7 supermotif-bearing peptides are, for example, engineered to possess a  
25 preferred residue (V, I, L, or F) at the C-terminal primary anchor position, as demonstrated by Sidney *et al. (J. Immunol. 157:3480-3490, 1996)*.

Analoging at primary anchor residues of other motif and/or supermotif-bearing epitopes is performed in a like manner.

30 The analog peptides are then be tested for immunogenicity, typically in a cellular screening assay. Again, it is generally important to demonstrate that analog-specific CTLs are also able to recognize the wild-type peptide and, when possible, targets that endogenously express the epitope.

#### Analoging at Secondary Anchor Residues

Moreover, HLA supermotifs are of value in engineering highly cross-reactive peptides and/or peptides that bind HLA molecules with increased affinity by identifying particular residues at  
35 secondary anchor positions that are associated with such properties. For example, the binding capacity of a B7 supermotif-bearing peptide with an F residue at position 1 is analyzed. The peptide is then



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analoged to, for example, substitute L for F at position 1. The analoged peptide is evaluated for increased binding affinity, binding half life and/or increased cross-reactivity. Such a procedure identifies analoged peptides with enhanced properties.

5 Engineered analogs with sufficiently improved binding capacity or cross-reactivity can also be tested for immunogenicity in HLA-B7-transgenic mice, following for example, IFA immunization or lipopeptide immunization. Analogued peptides are additionally tested for the ability to stimulate a recall response using PBMC from patients with 55P4H4-expressing tumors.

Other analoguing strategies

10 Another form of peptide analoguing, unrelated to anchor positions, involves the substitution of a cysteine with  $\alpha$ -amino butyric acid. Due to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally so as to reduce binding capacity. Substitution of  $\alpha$ -amino butyric acid for cysteine not only alleviates this problem, but has been shown to improve binding and crossbinding capabilities in some instances (see, e.g., the review by Sette *et al.*, In: Persistent Viral Infections, Eds. R. Ahmed and I. Chen, John Wiley & Sons, England, 1999).

15 Thus, by the use of single amino acid substitutions, the binding properties and/or cross-reactivity of peptide ligands for HLA supertype molecules can be modulated.

**Example 26: Identification of 55P4H4-derived Sequences with HLA-DR Binding Motifs**

20 Peptide epitopes bearing an HLA class II supermotif or motif are identified as outlined below using methodology similar to that described for HLA Class I peptides.

Selection of HLA-DR-supermotif-bearing epitopes.

25 To identify 55P4H4-derived, HLA class II HTL epitopes, the 55P4H4 antigen is analyzed for the presence of sequences bearing an HLA-DR-motif or supermotif. Specifically, 15-mer sequences are selected comprising a DR-supermotif, comprising a 9-mer core, and three-residue N- and C-terminal flanking regions (15 amino acids total).

30 Protocols for predicting peptide binding to DR molecules have been developed (Southwood *et al.*, *J. Immunol.* 160:3363-3373, 1998). These protocols, specific for individual DR molecules, allow the scoring, and ranking, of 9-mer core regions. Each protocol not only scores peptide sequences for the presence of DR-supermotif primary anchors (i.e., at position 1 and position 6) within a 9-mer core, but additionally evaluates sequences for the presence of secondary anchors. Using allele-specific selection tables (see, e.g., Southwood *et al.*, *ibid.*), it has been found that these protocols efficiently select peptide sequences with a high probability of binding a particular DR molecule. Additionally, it has been found that performing these protocols in tandem, specifically those for DR1, DR4w4, and DR7, can efficiently select DR cross-reactive peptides.

35 The 55P4H4-derived peptides identified above are tested for their binding capacity for various common HLA-DR molecules. All peptides are initially tested for binding to the DR molecules in the

primary panel: DR1, DR4w4, and DR7. Peptides binding at least two of these three DR molecules are then tested for binding to DR2w2  $\beta$ 1, DR2w2  $\beta$ 2, DR6w19, and DR9 molecules in secondary assays. Finally, peptides binding at least two of the four secondary panel DR molecules, and thus cumulatively at least four of seven different DR molecules, are screened for binding to DR4w15, DR5w11, and DR8w2 molecules in tertiary assays. Peptides binding at least seven of the ten DR molecules comprising the primary, secondary, and tertiary screening assays are considered cross-reactive DR binders. 55P4H4-derived peptides found to bind common HLA-DR alleles are of particular interest.

#### Selection of DR3 motif peptides

Because HLA-DR3 is an allele that is prevalent in Caucasian, Black, and Hispanic populations, DR3 binding capacity is a relevant criterion in the selection of HTL epitopes. Thus, peptides shown to be candidates may also be assayed for their DR3 binding capacity. However, in view of the binding specificity of the DR3 motif, peptides binding only to DR3 can also be considered as candidates for inclusion in a vaccine formulation.

To efficiently identify peptides that bind DR3, target 55P4H4 antigens are analyzed for sequences carrying one of the two DR3-specific binding motifs reported by Geluk *et al.* (*J. Immunol.* 152:5742-5748, 1994). The corresponding peptides are then synthesized and tested for the ability to bind DR3 with an affinity of 1  $\mu$ M or better, i.e., less than 1  $\mu$ M. Peptides are found that meet this binding criterion and qualify as HLA class II high affinity binders.

DR3 binding epitopes identified in this manner are included in vaccine compositions with DR supermotif-bearing peptide epitopes.

Similarly to the case of HLA class I motif-bearing peptides, the class II motif-bearing peptides are analoged to improve affinity or cross-reactivity. For example, aspartic acid at position 4 of the 9-mer core sequence is an optimal residue for DR3 binding, and substitution for that residue often improves DR 3 binding.

#### Example 27: Immunogenicity of 55P4H4-derived HTL epitopes

This example determines immunogenic DR supermotif- and DR3 motif-bearing epitopes among those identified using the methodology set forth herein.

Immunogenicity of HTL epitopes are evaluated in a manner analogous to the determination of immunogenicity of CTL epitopes, by assessing the ability to stimulate HTL responses and/or by using appropriate transgenic mouse models. Immunogenicity is determined by screening for: 1.) *in vitro* primary induction using normal PBMC or 2.) recall responses from patients who have 55P4H4-expressing tumors.

**Example 28: Calculation of phenotypic frequencies of HLA-supertypes in various ethnic backgrounds to determine breadth of population coverage**

This example illustrates the assessment of the breadth of population coverage of a vaccine composition comprised of multiple epitopes comprising multiple supermotifs and/or motifs.

5        In order to analyze population coverage, gene frequencies of HLA alleles are determined. Gene frequencies for each HLA allele are calculated from antigen or allele frequencies utilizing the binomial distribution formulae  $gf=1-(SQRT(1-af))$  (see, e.g., Sidney et al., Human Immunol. 45:79-93, 1996). To obtain overall phenotypic frequencies, cumulative gene frequencies are calculated, and the cumulative antigen frequencies derived by the use of the inverse formula  $[af=1-(1-Cgf)^2]$ .

10        Where frequency data is not available at the level of DNA typing, correspondence to the serologically defined antigen frequencies is assumed. To obtain total potential supertype population coverage no linkage disequilibrium is assumed, and only alleles confirmed to belong to each of the supertypes are included (minimal estimates). Estimates of total potential coverage achieved by inter-  
15        loci combinations are made by adding to the A coverage the proportion of the non-A covered population that could be expected to be covered by the B alleles considered (e.g.,  $total=A+B*(1-A)$ ). Confirmed members of the A3-like supertype are A3, A11, A31, A\*3301, and A\*6801. Although the A3-like supertype may also include A34, A66, and A\*7401, these alleles were not included in overall frequency calculations. Likewise, confirmed members of the A2-like supertype family are A\*0201, A\*0202, A\*0203, A\*0204, A\*0205, A\*0206, A\*0207, A\*6802, and A\*6901. Finally, the B7-like  
20        supertype-confirmed alleles are: B7, B\*3501-03, B51, B\*5301, B\*5401, B\*5501-2, B\*5601, B\*6701, and B\*7801 (potentially also B\*1401, B\*3504-06, B\*4201, and B\*5602).

Population coverage achieved by combining the A2-, A3- and B7-supertypes is approximately 86% in five major ethnic groups. Coverage may be extended by including peptides bearing the A1 and A24 motifs. On average, A1 is present in 12% and A24 in 29% of the population across five different  
25        major ethnic groups (Caucasian, North American Black, Chinese, Japanese, and Hispanic). Together, these alleles are represented with an average frequency of 39% in these same ethnic populations. The total coverage across the major ethnicities when A1 and A24 are combined with the coverage of the A2-, A3- and B7-supertype alleles is >95%. An analogous approach can be used to estimate population coverage achieved with combinations of class II motif-bearing epitopes.

30        Immunogenicity studies in humans (e.g., Bertoni et al., J. Clin. Invest. 100:503, 1997; Doolan et al., Immunity 7:97, 1997; and Threlkeld et al., J. Immunol. 159:1648, 1997) have shown that highly cross-reactive binding peptides are almost always recognized as epitopes. The use of highly cross-reactive binding peptides is an important selection criterion in identifying candidate epitopes for inclusion in a vaccine that is immunogenic in a diverse population.

35        With a sufficient number of epitopes (as disclosed herein and from the art), an average population coverage is predicted to be greater than 95% in each of five major ethnic populations. The game theory Monte Carlo simulation analysis, which is known in the art (see e.g., Osborne, M.J. and

Rubinstein, A. "A course in game theory" MIT Press, 1994), can be used to estimate what percentage of the individuals in a population comprised of the Caucasian, North American Black, Japanese, Chinese, and Hispanic ethnic groups would recognize the vaccine epitopes described herein. A preferred percentage is 90%. A more preferred percentage is 95%.

5

**Example 29: CTL Recognition of Endogenously Processed Antigens After Priming**

This example determines that CTL induced by native or analoged peptide epitopes identified and selected as described herein recognize endogenously synthesized, i.e., native antigens.

Effector cells isolated from transgenic mice that are immunized with peptide epitopes, for example HLA-A2 supermotif-bearing epitopes, are re-stimulated in vitro using peptide-coated stimulator cells. Six days later, effector cells are assayed for cytotoxicity and the cell lines that contain peptide-specific cytotoxic activity are further re-stimulated. An additional six days later, these cell lines are tested for cytotoxic activity on <sup>51</sup>Cr labeled Jurkat-A2.1/Kb target cells in the absence or presence of peptide, and also tested on <sup>51</sup>Cr labeled target cells bearing the endogenously synthesized antigen, i.e. cells that are stably transfected with 55P4H4 expression vectors.

The results demonstrate that CTL lines obtained from animals primed with peptide epitope recognize endogenously synthesized 55P4H4 antigen. The choice of transgenic mouse model to be used for such an analysis depends upon the epitope(s) that are being evaluated. In addition to HLA-A\*0201/Kb transgenic mice, several other transgenic mouse models including mice with human A11, which may also be used to evaluate A3 epitopes, and B7 alleles have been characterized and others (e.g., transgenic mice for HLA-A1 and A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed, which may be used to evaluate HTL epitopes.

**Example 30: Activity of CTL-HTL Conjugated Epitopes In Transgenic Mice**

This example illustrates the induction of CTLs and HTLs in transgenic mice, by use of a 55P4H4-derived CTL and HTL peptide vaccine compositions. The vaccine composition used herein comprise peptides to be administered to a patient with a 55P4H4-expressing tumor. The peptide composition can comprise multiple CTL and/or HTL epitopes. The epitopes are identified using methodology as described herein. This example also illustrates that enhanced immunogenicity can be achieved by inclusion of one or more HTL epitopes in a CTL vaccine composition; such a peptide composition can comprise an HTL epitope conjugated to a CTL epitope. The CTL epitope can be one that binds to multiple HLA family members at an affinity of 500 nM or less, or analogs of that epitope. The peptides may be lipidated, if desired.

Immunization procedures: Immunization of transgenic mice is performed as described (Alexander et al., J. Immunol. 159:4753-4761, 1997). For example, A2/Kb mice, which are transgenic for the human HLA A2.1 allele and are used to assess the immunogenicity of HLA-A\*0201 motif- or

HLA-A2 supermotif-bearing epitopes, and are primed subcutaneously (base of the tail) with a 0.1 ml of peptide in Incomplete Freund's Adjuvant, or if the peptide composition is a lipidated CTL/HTL conjugate, in DMSO/saline, or if the peptide composition is a polypeptide, in PBS or Incomplete Freund's Adjuvant. Seven days after priming, splenocytes obtained from these animals are  
5 restimulated with syngeneic irradiated LPS-activated lymphoblasts coated with peptide.

Cell lines: Target cells for peptide-specific cytotoxicity assays are Jurkat cells transfected with the HLA-A2.1/K<sup>b</sup> chimeric gene (e.g., Vitiello *et al.*, *J. Exp. Med.* 173:1007, 1991)

In vitro CTL activation: One week after priming, spleen cells ( $30 \times 10^6$  cells/flask) are co-cultured at 37°C with syngeneic, irradiated (3000 rads), peptide coated lymphoblasts ( $10 \times 10^6$   
10 cells/flask) in 10 ml of culture medium/T25 flask. After six days, effector cells are harvested and assayed for cytotoxic activity.

Assay for cytotoxic activity: Target cells ( $1.0$  to  $1.5 \times 10^6$ ) are incubated at 37°C in the presence of 200  $\mu$ l of <sup>51</sup>Cr. After 60 minutes, cells are washed three times and resuspended in R10 medium. Peptide is added where required at a concentration of 1  $\mu$ g/ml. For the assay, <sup>104</sup> <sup>51</sup>Cr-labeled target cells are added to different concentrations of effector cells (final volume of 200  $\mu$ l) in U-bottom 96-well plates. After a six hour incubation period at 37°C, a 0.1 ml aliquot of supernatant is removed from each well and radioactivity is determined in a Micromedic automatic gamma counter.  
15 The percent specific lysis is determined by the formula: percent specific release =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . To facilitate comparison  
20 between separate CTL assays run under the same conditions, % <sup>51</sup>Cr release data is expressed as lytic units/ $10^6$  cells. One lytic unit is arbitrarily defined as the number of effector cells required to achieve 30% lysis of 10,000 target cells in a six hour <sup>51</sup>Cr release assay. To obtain specific lytic units/ $10^6$ , the lytic units/ $10^6$  obtained in the absence of peptide is subtracted from the lytic units/ $10^6$  obtained in the presence of peptide. For example, if 30% <sup>51</sup>Cr release is obtained at the effector (E): target (T) ratio of  
25 50:1 (i.e.,  $5 \times 10^5$  effector cells for 10,000 targets) in the absence of peptide and 5:1 (i.e.,  $5 \times 10^4$  effector cells for 10,000 targets) in the presence of peptide, the specific lytic units would be:  $[(1/50,000) - (1/500,000)] \times 10^6 = 18 \text{ LU}$ .

The results are analyzed to assess the magnitude of the CTL responses of animals injected with the immunogenic CTL/HTL conjugate vaccine preparation and are compared to the magnitude of  
30 the CTL response achieved using, for example, CTL epitopes as outlined above in the Example entitled "Confirmation of Immunogenicity". Analyses similar to this may be performed to evaluate the immunogenicity of peptide conjugates containing multiple CTL epitopes and/or multiple HTL epitopes. In accordance with these procedures, it is found that a CTL response is induced, and concomitantly that an HTL response is induced upon administration of such compositions.

35

**Example 31: Selection of CTL and HTL Epitopes for Inclusion in a 55P4H4-specific Vaccine**

This example illustrates a procedure for selecting peptide epitopes for vaccine compositions of the invention. The peptides in the composition can be in the form of a nucleic acid sequence, either single or one or more sequences (i.e., minigene) that encodes peptide(s), or can be single and/or polyepitopic peptides.

The following principles are utilized when selecting a plurality of epitopes for inclusion in a vaccine composition. Each of the following principles is balanced in order to make the selection.

Epitopes are selected which, upon administration, mimic immune responses that are correlated with 55P4H4 clearance. The number of epitopes used depends on observations of patients who spontaneously clear 55P4H4. For example, if it has been observed that patients who spontaneously clear 55P4H4 generate an immune response to at least three (3) from 55P4H4 antigen, then three or four (3-4) epitopes should be included for HLA class I. A similar rationale is used to determine HLA class II epitopes.

Epitopes are often selected that have a binding affinity of an IC<sub>50</sub> of 500 nM or less for an HLA class I molecule, or for class II, an IC<sub>50</sub> of 1000 nM or less; or HLA Class I peptides with high binding scores from the BIMAS web site, <http://bimas.dcrf.nih.gov/>.

In order to achieve broad coverage of the vaccine through out a diverse population, sufficient supermotif bearing peptides, or a sufficient array of allele-specific motif bearing peptides, are selected to give broad population coverage. In one embodiment, epitopes are selected to provide at least 80% population coverage. A Monte Carlo analysis, a statistical evaluation known in the art, can be employed to assess breadth, or redundancy, of population coverage.

When creating polyepitopic compositions, or a minigene that encodes same, it is typically desirable to generate the smallest peptide possible that encompasses the epitopes of interest. The principles employed are similar, if not the same, as those employed when selecting a peptide comprising nested epitopes. For example, a protein sequence for the vaccine composition is selected because it has maximal number of epitopes contained within the sequence, i.e., it has a high concentration of epitopes. Epitopes may be nested or overlapping (i.e., frame shifted relative to one another). For example, with overlapping epitopes, two 9-mer epitopes and one 10-mer epitope can be present in a 10 amino acid peptide. Each epitope can be exposed and bound by an HLA molecule upon administration of such a peptide. A multi-epitopic, peptide can be generated synthetically, recombinantly, or via cleavage from the native source. Alternatively, an analog can be made of this native sequence, whereby one or more of the epitopes comprise substitutions that alter the cross-reactivity and/or binding affinity properties of the polyepitopic peptide. Such a vaccine composition is administered for therapeutic or prophylactic purposes. This embodiment provides for the possibility that an as yet undiscovered aspect of immune system processing will apply to the native nested sequence and thereby facilitate the production of therapeutic or prophylactic immune response-

inducing vaccine compositions. Additionally such an embodiment provides for the possibility of motif-bearing epitopes for an HLA makeup that is presently unknown. Furthermore, this embodiment (absent the creating of any analogs) directs the immune response to multiple peptide sequences that are actually present in 55P4H4, thus avoiding the need to evaluate any junctional epitopes. Lastly, the  
5 embodiment provides an economy of scale when producing nucleic acid vaccine compositions. Related to this embodiment, computer programs can be derived in accordance with principles in the art, which identify in a target sequence, the greatest number of epitopes per sequence length.

A vaccine composition comprised of selected peptides, when administered, is safe, efficacious, and elicits an immune response similar in magnitude to an immune response that controls  
10 or clears cells that bear or overexpress 55P4H4.

### **Example 32: Construction of "Minigene" Multi-Epitope DNA Plasmids**

This example discusses the construction of a minigene expression plasmid. Minigene plasmids may, of course, contain various configurations of B cell, CTL and/or HTL epitopes or epitope  
15 analogs as described herein.

A minigene expression plasmid typically includes multiple CTL and HTL peptide epitopes. In the present example, HLA-A2, -A3, -B7 supermotif-bearing peptide epitopes and HLA-A1 and -A24 motif-bearing peptide epitopes are used in conjunction with DR supermotif-bearing epitopes and/or DR3 epitopes. HLA class I supermotif or motif-bearing peptide epitopes derived 55P4H4, are selected  
20 such that multiple supermotifs/motifs are represented to ensure broad population coverage. Similarly, HLA class II epitopes are selected from 55P4H4 to provide broad population coverage, *i.e.* both HLA DR-1-4-7 supermotif-bearing epitopes and HLA DR-3 motif-bearing epitopes are selected for inclusion in the minigene construct. The selected CTL and HTL epitopes are then incorporated into a minigene for expression in an expression vector.

Such a construct may additionally include sequences that direct the HTL epitopes to the endoplasmic reticulum. For example, the Ii protein may be fused to one or more HTL epitopes as described in the art, wherein the CLIP sequence of the Ii protein is removed and replaced with an HLA class II epitope sequence so that HLA class II epitope is directed to the endoplasmic reticulum, where the epitope binds to an HLA class II molecules.  
25

This example illustrates the methods to be used for construction of a minigene-bearing expression plasmid. Other expression vectors that may be used for minigene compositions are available and known to those of skill in the art.  
30

The minigene DNA plasmid of this example contains a consensus Kozak sequence and a consensus murine kappa Ig-light chain signal sequence followed by CTL and/or HTL epitopes selected  
35 in accordance with principles disclosed herein. The sequence encodes an open reading frame fused to the Myc and His antibody epitope tag coded for by the pcDNA 3.1 Myc-His vector.

Overlapping oligonucleotides that can, for example, average about 70 nucleotides in length with 15 nucleotide overlaps, are synthesized and HPLC-purified. The oligonucleotides encode the selected peptide epitopes as well as appropriate linker nucleotides, Kozak sequence, and signal sequence. The final multiepitope minigene is assembled by extending the overlapping oligonucleotides in three sets of reactions using PCR. A Perkin/Elmer 9600 PCR machine is used and a total of 30 cycles are performed using the following conditions: 95°C for 15 sec, annealing temperature (5° below the lowest calculated T<sub>m</sub> of each primer pair) for 30 sec, and 72°C for 1 min.

For example, a minigene is prepared as follows. For a first PCR reaction, 5 µg of each of two oligonucleotides are annealed and extended: In an example using eight oligonucleotides, i.e., four pairs of primers, oligonucleotides 1+2, 3+4, 5+6, and 7+8 are combined in 100 µl reactions containing *Pfu* polymerase buffer (1x= 10 mM KCL, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-chloride, pH 8.75, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 100 µg/ml BSA), 0.25 mM each dNTP, and 2.5 U of *Pfu* polymerase. The full-length dimer-products are gel-purified, and two reactions containing the product of 1+2 and 3+4, and the product of 5+6 and 7+8 are mixed, annealed, and extended for 10 cycles. Half of the two reactions are then mixed, and 5 cycles of annealing and extension carried out before flanking primers are added to amplify the full length product. The full-length product is gel-purified and cloned into pCR-blunt (Invitrogen) and individual clones are screened by sequencing.

### **Example 33: The Plasmid Construct and the Degree to Which It Induces Immunogenicity**

The degree to which a plasmid construct, for example a plasmid constructed in accordance with the previous Example, is able to induce immunogenicity is evaluated *in vitro* by testing for epitope presentation by APC following transduction or transfection of the APC with an epitope-expressing nucleic acid construct. Such a study determines "antigenicity" and allows the use of human APC. The assay determines the ability of the epitope to be presented by the APC in a context that is recognized by a T cell by quantifying the density of epitope-HLA class I complexes on the cell surface. Quantitation can be performed by directly measuring the amount of peptide eluted from the APC (see, e.g., Sijts *et al.*, *J. Immunol.* 156:683-692, 1996; Demotz *et al.*, *Nature* 342:682-684, 1989); or the number of peptide-HLA class I complexes can be estimated by measuring the amount of lysis or lymphokine release induced by diseased or transfected target cells, and then determining the concentration of peptide necessary to obtain equivalent levels of lysis or lymphokine release (see, e.g., Kageyama *et al.*, *J. Immunol.* 154:567-576, 1995).

Alternatively, immunogenicity is evaluated through *in vivo* injections into mice and subsequent *in vitro* assessment of CTL and HTL activity, which are analyzed using cytotoxicity and proliferation assays, respectively, as detailed e.g., in Alexander *et al.*, *Immunity* 1:751-761, 1994.

For example, to assess the capacity of a DNA minigene construct containing at least one HLA-A2 supermotif peptide to induce CTLs *in vivo*, HLA-A2.1/K<sup>b</sup> transgenic mice, for example, are



immunized intramuscularly with 100 µg of naked cDNA. As a means of comparing the level of CTLs induced by cDNA immunization, a control group of animals is also immunized with an actual peptide composition that comprises multiple epitopes synthesized as a single polypeptide as they would be encoded by the minigene.

5           Splenocytes from immunized animals are stimulated twice with each of the respective compositions (peptide epitopes encoded in the minigene or the polyepitopic peptide), then assayed for peptide-specific cytotoxic activity in a <sup>51</sup>Cr release assay. The results indicate the magnitude of the CTL response directed against the A2-restricted epitope, thus indicating the *in vivo* immunogenicity of the minigene vaccine and polyepitopic vaccine.

10           It is, therefore, found that the minigene elicits immune responses directed toward the HLA-A2 supermotif peptide epitopes as does the polyepitopic peptide vaccine. A similar analysis is also performed using other HLA-A3 and HLA-B7 transgenic mouse models to assess CTL induction by HLA-A3 and HLA-B7 motif or supermotif epitopes, whereby it is also found that the minigene elicits appropriate immune responses directed toward the provided epitopes.

15           To assess the capacity of a class II epitope-encoding minigene to induce HTLs *in vivo*, DR transgenic mice, or for those epitopes that cross react with the appropriate mouse MHC molecule, I-Ab-restricted mice, for example, are immunized intramuscularly with 100 µg of plasmid DNA. As a means of comparing the level of HTLs induced by DNA immunization, a group of control animals is also immunized with an actual peptide composition emulsified in complete Freund's adjuvant. CD4+  
20   T cells, *i.e.* HTLs, are purified from splenocytes of immunized animals and stimulated with each of the respective compositions (peptides encoded in the minigene). The HTL response is measured using a <sup>3</sup>H-thymidine incorporation proliferation assay, (*see, e.g.,* Alexander et al. *Immunity* 1:751-761, 1994). The results indicate the magnitude of the HTL response, thus demonstrating the *in vivo* immunogenicity of the minigene.

25           DNA minigenes, constructed as described in the previous Example, can also be evaluated as a vaccine in combination with a boosting agent using a prime boost protocol. The boosting agent can consist of recombinant protein (*e.g.,* Barnett *et al., Aids Res. and Human Retroviruses* 14, Supplement 3:S299-S309, 1998) or recombinant vaccinia, for example, expressing a minigene or DNA encoding the complete protein of interest (*see, e.g.,* Hanke *et al., Vaccine* 16:439-445, 1998; Sedegah *et al., Proc. Natl. Acad. Sci USA* 95:7648-53, 1998; Hanke and McMichael, *Immunol. Letters* 66:177-181, 1999; and Robinson et al., *Nature Med.* 5:526-34, 1999).  
30

          For example, the efficacy of the DNA minigene used in a prime boost protocol is initially evaluated in transgenic mice. In this example, A2.1/Kb transgenic mice are immunized IM with 100 µg of a DNA minigene encoding the immunogenic peptides including at least one HLA-A2 supermotif-bearing peptide. After an incubation period (ranging from 3-9 weeks), the mice are boosted IP with  
35   107 pfu/mouse of a recombinant vaccinia virus expressing the same sequence encoded by the DNA

minigene. Control mice are immunized with 100 µg of DNA or recombinant vaccinia without the minigene sequence, or with DNA encoding the minigene, but without the vaccinia boost. After an additional incubation period of two weeks, splenocytes from the mice are immediately assayed for peptide-specific activity in an ELISPOT assay. Additionally, splenocytes are stimulated in vitro with the A2-restricted peptide epitopes encoded in the minigene and recombinant vaccinia, then assayed for peptide-specific activity in an alpha, beta and/or gamma IFN ELISA.

It is found that the minigene utilized in a prime-boost protocol elicits greater immune responses toward the HLA-A2 supermotif peptides than with DNA alone. Such an analysis can also be performed using HLA-A11 or HLA-B7 transgenic mouse models to assess CTL induction by HLA-A3 or HLA-B7 motif or supermotif epitopes. The use of prime boost protocols in humans is described below in the Example entitled "Induction of CTL Responses Using a Prime Boost Protocol."

#### **Example 34: Peptide Composition for Prophylactic Uses**

Vaccine compositions of the present invention can be used to prevent 55P4H4 expression in persons who are at risk for tumors that bear this antigen. For example, a polypeptidic peptide epitope composition (or a nucleic acid comprising the same) containing multiple CTL and HTL epitopes such as those selected in the above Examples, which are also selected to target greater than 80% of the population, is administered to individuals at risk for a 55P4H4-associated tumor.

For example, a peptide-based composition is provided as a single polypeptide that encompasses multiple epitopes. The vaccine is typically administered in a physiological solution that comprises an adjuvant, such as Incomplete Freund's Adjuvant. The dose of peptide for the initial immunization is from about 1 to about 50,000 µg, generally 100-5,000 µg, for a 70 kg patient. The initial administration of vaccine is followed by booster dosages at 4 weeks followed by evaluation of the magnitude of the immune response in the patient, by techniques that determine the presence of epitope-specific CTL populations in a PBMC sample. Additional booster doses are administered as required. The composition is found to be both safe and efficacious as a prophylaxis against 55P4H4-associated disease.

Alternatively, a composition typically comprising transfecting agents is used for the administration of a nucleic acid-based vaccine in accordance with methodologies known in the art and disclosed herein.

#### **Example 35: Polypeptidic Vaccine Compositions Derived from Native 55P4H4 Sequences**

A native 55P4H4 polyprotein sequence is screened, preferably using computer algorithms defined for each class I and/or class II supermotif or motif, to identify "relatively short" regions of the polyprotein that comprise multiple epitopes. The "relatively short" regions are preferably less in length

than an entire native antigen. This relatively short sequence that contains multiple distinct or overlapping, "nested" epitopes is selected; it can be used to generate a minigene construct. The construct is engineered to express the peptide, which corresponds to the native protein sequence. The "relatively short" peptide is generally less than 250 amino acids in length, often less than 100 amino acids in length, preferably less than 75 amino acids in length, and more preferably less than 50 amino acids in length. The protein sequence of the vaccine composition is selected because it has maximal number of epitopes contained within the sequence, *i.e.*, it has a high concentration of epitopes. As noted herein, epitope motifs may be nested or overlapping (*i.e.*, frame shifted relative to one another). For example, with overlapping epitopes, two 9-mer epitopes and one 10-mer epitope can be present in a 10 amino acid peptide. Such a vaccine composition is administered for therapeutic or prophylactic purposes.

The vaccine composition will include, for example, multiple CTL epitopes from 55P4H4 antigen and at least one HTL epitope. This polyepitopic native-sequence is administered either as a peptide or as a nucleic acid sequence which encodes the peptide. Alternatively, an analog can be made of this native sequence, whereby one or more of the epitopes comprise substitutions that alter the cross-reactivity and/or binding affinity properties of the polyepitopic peptide.

The embodiment of this example provides for the possibility that an as yet undiscovered aspect of immune system processing will apply to the native nested sequence and thereby facilitate the production of therapeutic or prophylactic immune response-inducing vaccine compositions. Additionally such an embodiment provides for the possibility of motif-bearing epitopes for an HLA makeup that is presently unknown. Furthermore, this embodiment (excluding an analoged embodiment) directs the immune response to multiple peptide sequences that are actually present in native 55P4H4, thus avoiding the need to evaluate any junctional epitopes. Lastly, the embodiment provides an economy of scale when producing peptide or nucleic acid vaccine compositions.

Related to this embodiment, computer programs are available in the art which can be used to identify in a target sequence, the greatest number of epitopes per sequence length.

#### **Example 36: Polyepitopic Vaccine Compositions From Multiple Antigens**

The 55P4H4 peptide epitopes of the present invention are used in conjunction with epitopes from other target tumor-associated antigens, to create a vaccine composition that is useful for the prevention or treatment of cancer that expresses 55P4H4 and such other antigens. For example, a vaccine composition can be provided as a single polypeptide that incorporates multiple epitopes from 55P4H4 as well as tumor-associated antigens that are often expressed with a target cancer associated with 55P4H4 expression, or can be administered as a composition comprising a cocktail of one or more discrete epitopes. Alternatively, the vaccine can be administered as a minigene construct or as dendritic cells which have been loaded with the peptide epitopes *in vitro*.

### **Example 37: Use of Peptides to Evaluate an Immune Response**

Peptides of the invention may be used to analyze an immune response for the presence of specific antibodies, CTL or HTL directed to 55P4H4. Such an analysis can be performed in a manner described by Ogg *et al.*, *Science* 279:2103-2106, 1998. In this Example, peptides in accordance with the invention are used as a reagent for diagnostic or prognostic purposes, not as an immunogen.

In this example highly sensitive human leukocyte antigen tetrameric complexes ("tetramers") are used for a cross-sectional analysis of, for example, 55P4H4 HLA-A\*0201-specific CTL frequencies from HLA A\*0201-positive individuals at different stages of disease or following immunization comprising a 55P4H4 peptide containing an A\*0201 motif. Tetrameric complexes are synthesized as described (Musey *et al.*, *N. Engl. J. Med.* 337:1267, 1997). Briefly, purified HLA heavy chain (A\*0201 in this example) and  $\beta$ 2-microglobulin are synthesized by means of a prokaryotic expression system. The heavy chain is modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a sequence containing a BirA enzymatic biotinylation site. The heavy chain,  $\beta$ 2-microglobulin, and peptide are refolded by dilution. The 45-kD refolded product is isolated by fast protein liquid chromatography and then biotinylated by BirA in the presence of biotin (Sigma, St. Louis, Missouri), adenosine 5'triphosphate and magnesium. Streptavidin-phycoerythrin conjugate is added in a 1:4 molar ratio, and the tetrameric product is concentrated to 1 mg/ml. The resulting product is referred to as tetramer-phycoerythrin.

For the analysis of patient blood samples, approximately one million PBMCs are centrifuged at 300g for 5 minutes and resuspended in 50  $\mu$ l of cold phosphate-buffered saline. Tri-color analysis is performed with the tetramer-phycoerythrin, along with anti-CD8-Tricolor, and anti-CD38. The PBMCs are incubated with tetramer and antibodies on ice for 30 to 60 min and then washed twice before formaldehyde fixation. Gates are applied to contain >99.98% of control samples. Controls for the tetramers include both A\*0201-negative individuals and A\*0201-positive non-diseased donors. The percentage of cells stained with the tetramer is then determined by flow cytometry. The results indicate the number of cells in the PBMC sample that contain epitope-restricted CTLs, thereby readily indicating the extent of immune response to the 55P4H4 epitope, and thus the status of exposure to 55P4H4, or exposure to a vaccine that elicits a protective or therapeutic response.

### **Example 38: Use of Peptide Epitopes to Evaluate Recall Responses**

The peptide epitopes of the invention are used as reagents to evaluate T cell responses, such as acute or recall responses, in patients. Such an analysis may be performed on patients who have recovered from 55P4H4-associated disease or who have been vaccinated with a 55P4H4 vaccine.

For example, the class I restricted CTL response of persons who have been vaccinated may be analyzed. The vaccine may be any 55P4H4 vaccine. PBMC are collected from vaccinated individuals and HLA typed. Appropriate peptide epitopes of the invention that, optimally, bear supermotifs to provide cross-reactivity with multiple HLA supertype family members, are then used for analysis of samples derived from individuals who bear that HLA type.

PBMC from vaccinated individuals are separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (GIBCO Laboratories), resuspended in RPMI-1640 (GIBCO Laboratories) supplemented with L-glutamine (2mM), penicillin (50U/ml), streptomycin (50 µg/ml), and Hepes (10mM) containing 10% heat-inactivated human AB serum (complete RPMI) and plated using microculture formats. A synthetic peptide comprising an epitope of the invention is added at 10 µg/ml to each well and HBV core 128-140 epitope is added at 1 µg/ml to each well as a source of T cell help during the first week of stimulation.

In-the-microculture-format,  $4 \times 10^5$  PBMC are stimulated with peptide in 8 replicate cultures in 96-well round bottom plate in 100 µl/well of complete RPMI. On days 3 and 10, 100 µl of complete RPMI and 20 U/ml final concentration of rIL-2 are added to each well. On day 7 the cultures are transferred into a 96-well flat-bottom plate and restimulated with peptide, rIL-2 and  $10^5$  irradiated (3,000 rad) autologous feeder cells. The cultures are tested for cytotoxic activity on day 14. A positive CTL response requires two or more of the eight replicate cultures to display greater than 10% specific  $^{51}\text{Cr}$  release, based on comparison with non-diseased control subjects as previously described (Rehermann, *et al.*, *Nature Med.* 2:1104,1108, 1996; Rehermann *et al.*, *J. Clin. Invest.* 97:1655-1665, 1996; and Rehermann *et al.* *J. Clin. Invest.* 98:1432-1440, 1996).

Target cell lines are autologous and allogeneic EBV-transformed B-LCL that are either purchased from the American Society for Histocompatibility and Immunogenetics (ASHI, Boston, MA) or established from the pool of patients as described (Guilhot, *et al.* *J. Virol.* 66:2670-2678, 1992).

Cytotoxicity assays are performed in the following manner. Target cells consist of either allogeneic HLA-matched or autologous EBV-transformed B lymphoblastoid cell line that are incubated overnight with the synthetic peptide epitope of the invention at 10 µM, and labeled with 100 µCi of  $^{51}\text{Cr}$  (Amersham Corp., Arlington Heights, IL) for 1 hour after which they are washed four times with HBSS.

Cytolytic activity is determined in a standard 4-h, split well  $^{51}\text{Cr}$  release assay using U-bottomed 96 well plates containing 3,000 targets/well. Stimulated PBMC are tested at effector/target (E/T) ratios of 20-50:1 on day 14. Percent cytotoxicity is determined from the formula:  $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$ . Maximum release is determined by lysis of targets by detergent (2% Triton X-100; Sigma Chemical Co., St. Louis, MO). Spontaneous release is <25% of maximum release for all experiments.

The results of such an analysis indicate the extent to which HLA-restricted CTL populations have been stimulated by previous exposure to 55P4H4 or a 55P4H4 vaccine.

Similarly, Class II restricted HTL responses may also be analyzed. Purified PBMC are cultured in a 96-well flat bottom plate at a density of  $1.5 \times 10^5$  cells/well and are stimulated with 10  $\mu\text{g/ml}$  synthetic peptide of the invention, whole 55P4H4 antigen, or PHA. Cells are routinely plated in replicates of 4-6 wells for each condition. After seven days of culture, the medium is removed and replaced with fresh medium containing 10U/ml IL-2. Two days later, 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine is added to each well and incubation is continued for an additional 18 hours. Cellular DNA is then harvested on glass fiber mats and analyzed for  $^3\text{H}$ -thymidine incorporation. Antigen-specific T cell proliferation is calculated as the ratio of  $^3\text{H}$ -thymidine incorporation in the presence of antigen divided by the  $^3\text{H}$ -thymidine incorporation in the absence of antigen.

#### **Example 39: Induction of Specific CTL Response In Humans**

A human clinical trial for an immunogenic composition comprising CTL and HTL epitopes of the invention is set up as an IND Phase I, dose escalation study and carried out as a randomized, double-blind, placebo-controlled trial. Such a trial is designed, for example, as follows:

A total of about 27 individuals are enrolled and divided into 3 groups:

Group I: 3 subjects are injected with placebo and 6 subjects are injected with 5  $\mu\text{g}$  of peptide composition;

Group II: 3 subjects are injected with placebo and 6 subjects are injected with 50  $\mu\text{g}$  peptide composition;

Group III: 3 subjects are injected with placebo and 6 subjects are injected with 500  $\mu\text{g}$  of peptide composition.

After 4 weeks following the first injection, all subjects receive a booster inoculation at the same dosage.

The endpoints measured in this study relate to the safety and tolerability of the peptide composition as well as its immunogenicity. Cellular immune responses to the peptide composition are an index of the intrinsic activity of this the peptide composition, and can therefore be viewed as a measure of biological efficacy. The following summarize the clinical and laboratory data that relate to safety and efficacy endpoints.

**Safety:** The incidence of adverse events is monitored in the placebo and drug treatment group and assessed in terms of degree and reversibility.

**Evaluation of Vaccine Efficacy:** For evaluation of vaccine efficacy, subjects are bled before and after injection. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation, aliquoted in freezing media and stored frozen. Samples are assayed for CTL and HTL activity.

The vaccine is found to be both safe and efficacious.

**Example 40: Phase II Trials In Patients Expressing 55P4H4**

5 Phase II trials are performed to study the effect of administering the CTL-HTL peptide compositions to patients having cancer that expresses 55P4H4. The main objectives of the trial are to determine an effective dose and regimen for inducing CTLs in cancer patients that express 55P4H4, to establish the safety of inducing a CTL and HTL response in these patients, and to see to what extent activation of CTLs improves the clinical picture of these patients, as manifested, e.g., by the reduction and/or shrinking of lesions. Such a study is designed, for example, as follows:

10 The studies are performed in multiple centers. The trial design is an open-label, uncontrolled, dose escalation protocol wherein the peptide composition is administered as a single dose followed six weeks later by a single booster shot of the same dose. The dosages are 50, 500 and 5,000 micrograms per injection. Drug-associated adverse effects (severity and reversibility) are recorded.

15 There are three patient groupings. The first group is injected with 50 micrograms of the peptide composition and the second and third groups with 500 and 5,000 micrograms of peptide composition, respectively. The patients within each group range in age from 21-65 and represent diverse ethnic backgrounds. All of them have a tumor that expresses 55P4H4.

20 Clinical manifestations or antigen-specific T-cell responses are monitored to assess the effects of administering the peptide compositions. The vaccine composition is found to be both safe and efficacious in the treatment of 55P4H4-associated disease.

**Example 41: Induction of CTL Responses Using a Prime Boost Protocol**

25 A prime boost protocol similar in its underlying principle to that used to evaluate the efficacy of a DNA vaccine in transgenic mice, such as described above in the Example entitled "The Plasmid Construct and the Degree to Which It Induces Immunogenicity," can also be used for the administration of the vaccine to humans. Such a vaccine regimen can include an initial administration of, for example, naked DNA followed by a boost using recombinant virus encoding the vaccine, or recombinant protein/polypeptide or a peptide mixture administered in an adjuvant.

30 For example, the initial immunization may be performed using an expression vector, such as that constructed in the Example entitled "Construction of 'Minigene' Multi-Epitope DNA Plasmids" in the form of naked nucleic acid administered IM (or SC or ID) in the amounts of 0.5-5 mg at multiple sites. The nucleic acid (0.1 to 1000 µg) can also be administered using a gene gun. Following an incubation period of 3-4 weeks, a booster dose is then administered. The booster can be recombinant fowlpox virus administered at a dose of  $5 \cdot 10^7$  to  $5 \cdot 10^9$  pfu. An alternative recombinant virus, such as  
35 an MVA, canarypox, adenovirus, or adeno-associated virus, can also be used for the booster, or the

polyepitopic protein or a mixture of the peptides can be administered. For evaluation of vaccine efficacy, patient blood samples are obtained before immunization as well as at intervals following administration of the initial vaccine and booster doses of the vaccine. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation, aliquoted in freezing media and stored frozen. Samples are assayed for CTL and HTL activity.

Analysis of the results indicates that a magnitude of response sufficient to achieve a therapeutic or protective immunity against 55P4H4 is generated.

#### **Example 42: Administration of Vaccine Compositions Using Dendritic Cells (DC)**

Vaccines comprising peptide epitopes of the invention can be administered using APCs, or "professional" APCs such as DC. In this example, peptide-pulsed DC are administered to a patient to stimulate a CTL response *in vivo*. In this method, dendritic cells are isolated, expanded, and pulsed with a vaccine comprising peptide CTL and HTL epitopes of the invention. The dendritic cells are infused back into the patient to elicit CTL and HTL responses *in vivo*. The induced CTL and HTL then destroy or facilitate destruction, respectively, of the target cells that bear the 55P4H4 protein from which the epitopes in the vaccine are derived.

For example, a cocktail of epitope-comprising peptides is administered *ex vivo* to PBMC, or isolated DC therefrom. A pharmaceutical to facilitate harvesting of DC can be used, such as Progenipoiectin™ (Monsanto, St. Louis, MO) or GM-CSF/IL-4. After pulsing the DC with peptides, and prior to reinfusion into patients, the DC are washed to remove unbound peptides.

As appreciated clinically, and readily determined by one of skill based on clinical outcomes, the number of DC reinfused into the patient can vary (*see, e.g., Nature Med.* 4:328, 1998; *Nature Med.* 2:52, 1996 and *Prostate* 32:272, 1997). Although  $2-50 \times 10^6$  DC per patient are typically administered, larger number of DC, such as  $10^7$  or  $10^8$  can also be provided. Such cell populations typically contain between 50-90% DC.

In some embodiments, peptide-loaded PBMC are injected into patients without purification of the DC. For example, PBMC generated after treatment with an agent such as Progenipoiectin™ are injected into patients without purification of the DC. The total number of PBMC that are administered often ranges from  $10^8$  to  $10^{10}$ . Generally, the cell doses injected into patients is based on the percentage of DC in the blood of each patient, as determined, for example, by immunofluorescence analysis with specific anti-DC antibodies. Thus, for example, if Progenipoiectin™ mobilizes 2% DC in the peripheral blood of a given patient, and that patient is to receive  $5 \times 10^6$  DC, then the patient will be injected with a total of  $2.5 \times 10^8$  peptide-loaded PBMC. The percent DC mobilized by an agent such as Progenipoiectin™ is typically estimated to be between 2-10%, but can vary as appreciated by one of skill in the art.

#### **Ex vivo activation of CTL/HTL responses**



Alternatively, *ex vivo* CTL or HTL responses to 55P4H4 antigens can be induced by incubating, in tissue culture, the patient's, or genetically compatible, CTL or HTL precursor cells together with a source of APC, such as DC, and immunogenic peptides. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded  
5 into effector cells, the cells are infused into the patient, where they will destroy (CTL) or facilitate destruction (HTL) of their specific target cells, *i.e.*, tumor cells.

**Example 43: Alternative Method of Identifying Motif-Bearing Peptides**

Another method of identifying motif-bearing peptides is to elute them from cells bearing  
10 defined MHC molecules. For example, EBV transformed B cell lines used for tissue typing have been extensively characterized to determine which HLA molecules they express. In certain cases these cells express only a single type of HLA molecule. These cells can be transfected with nucleic acids that express the antigen of interest, *e.g.* 55P4H4. Peptides produced by endogenous antigen processing of peptides produced as a result of transfection will then bind to HLA molecules within the cell and be  
15 transported and displayed on the cell's surface. Peptides are then eluted from the HLA molecules by exposure to mild acid conditions and their amino acid sequence determined, *e.g.*, by mass spectral analysis (*e.g.*, Kubo *et al.*, *J. Immunol.* 152:3913, 1994). Because the majority of peptides that bind a particular HLA molecule are motif-bearing, this is an alternative modality for obtaining the motif-bearing peptides correlated with the particular HLA molecule expressed on the cell.

20 Alternatively, cell lines that do not express endogenous HLA molecules can be transfected with an expression construct encoding a single HLA allele. These cells can then be used as described, *i.e.*, they can then be transfected with nucleic acids that encode 55P4H4 to isolate peptides corresponding to 55P4H4 that have been presented on the cell surface. Peptides obtained from such an analysis will bear motif(s) that correspond to binding to the single HLA allele that is expressed in the  
25 cell.

As appreciated by one in the art, one can perform a similar analysis on a cell bearing more than one HLA allele and subsequently determine peptides specific for each HLA allele expressed. Moreover, one of skill would also recognize that means other than transfection, such as loading with a protein antigen, can be used to provide a source of antigen to the cell.  
30

**Example 44: Complementary Polynucleotides**

Sequences complementary to the 55P4H4-encoding sequences, or any parts thereof, are used to detect, decrease, or inhibit expression of naturally occurring 55P4H4. Although use of oligonucleotides comprising from about 15 to 30 base pairs is described, essentially the same procedure  
35 is used with smaller or with larger sequence fragments. Appropriate oligonucleotides are designed using, *e.g.*, OLIGO 4.06 software (National Biosciences) and the coding sequence of 55P4H4. To

inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the 5SP4H4-encoding transcript.

5     **Example 45: Purification of Naturally-occurring or Recombinant 5SP4H4 Using 5SP4H4 Specific Antibodies**

          Naturally occurring or recombinant 5SP4H4 is substantially purified by immunoaffinity chromatography using antibodies specific for 5SP4H4. An immunoaffinity column is constructed by covalently coupling anti-5SP4H4 antibody to an activated chromatographic resin, such as CNBr-activated SEPHAROSE (Amersham Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

          Media containing 5SP4H4 are passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of 5SP4H4 (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/5SP4H4 binding (e.g., a buffer of pH 2 to pH 3, or a high concentration of a chaotrope, such as urea or thiocyanate ion), and GCR.P is collected.

**Example 46: Identification of Molecules Which Interact with 5SP4H4**

          5SP4H4, or biologically active fragments thereof, are labeled with 121 1 Bolton-Hunter reagent. (See, e.g., Bolton et al. (1973) Biochem. J. 133:529.) Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled 5SP4H4, washed, and any wells with labeled 5SP4H4 complex are assayed. Data obtained using different concentrations of 5SP4H4 are used to calculate values for the number, affinity, and association of 5SP4H4 with the candidate molecules.

25

          Throughout this application, various publications and applications are referenced. The disclosures of these publications and applications are hereby incorporated by reference herein in their entireties.

30

          The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any that are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope

35

of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

## TABLES

**TABLE I: Tissues that Express 55P4H4 When Malignant**

Prostate	Bone
Kidney	Bladder
Testis	Brain
Lung	Ovary
Cervix	

**TABLE II: AMINO ACID ABBREVIATIONS**

SINGLE LETTER	THREE LETTER	FULL NAME
F	Phe	phenylalanine
L	Leu	leucine
S	Ser	serine
Y	Tyr	tyrosine
C	Cys	cysteine
W	Trp	tryptophan
P	Pro	proline
H	His	histidine
Q	Gln	glutamine
R	Arg	arginine
I	Ile	isoleucine
M	Met	methionine
T	Thr	threonine
N	Asn	asparagine
K	Lys	lysine
V	Val	valine
A	Ala	alanine
D	Asp	aspartic acid
E	Glu	glutamic acid
G	Gly	glycine

**TABLE III: AMINO ACID SUBSTITUTION MATRIX**

Adapted from the GCG Software 9.0 BLOSUM62 amino acid substitution matrix (block substitution matrix). The higher the value, the more likely a substitution is found in related, natural proteins.

A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
4	0	-2	-1	-2	0	-2	-1	-1	-1	-1	-2	-1	-1	-1	1	0	0	-3	-2	A
	9	-3	-4	-2	-3	-3	-1	-3	-1	-1	-3	-3	-3	-3	-1	-1	-1	-2	-2	C
		6	2	-3	-1	-1	-3	-1	-4	-3	1	-1	0	-2	0	-1	-3	-4	-3	D
			5	-3	-2	0	-3	1	-3	-2	0	-1	2	0	0	-1	-2	-3	-2	E
				6	-3	-1	0	-3	0	0	-3	-4	-3	-3	-2	-2	-1	1	3	F
					6	-2	-4	-2	-4	-3	0	-2	-2	-2	0	-2	-3	-2	-3	G
						8	-3	-1	-3	-2	1	-2	0	0	-1	-2	-3	-2	2	H
							4	-3	2	1	-3	-3	-3	-3	-2	-1	3	-3	-1	I
								5	-2	-1	0	-1	1	2	0	-1	-2	-3	-2	K
									4	2	-3	-3	-2	-2	-2	-1	1	-2	-1	L
										5	-2	-2	0	-1	-1	-1	1	-1	-1	M
											6	-2	0	0	1	0	-3	-4	-2	N
												7	-1	-2	-1	-1	-2	-4	-3	P
													5	1	0	-1	-2	-2	-1	Q
														5	-1	-1	-3	-3	-2	R
															4	1	-2	-3	-2	S
																5	0	-2	-2	T
																	4	-3	-1	V
																		11	2	W
																			7	Y

**TABLE IV (A): HLA CLASS I SUPERMOTIFS**

SUPERMOTIF	POSITION 2	C-TERMINUS
A2	L, I, V, M, A, T, Q	L, I, V, M, A, T
A3	A, V, I, L, M, S, T	R, K
B7	P	A, L, I, M, V, F, W, Y
B44	D, E	F, W, Y, L, I, M, V, A
A1	T, S, L, I, V, M	F, W, Y
A24	F, W, Y, L, V, I, M, T	F, I, Y, W, L, M
B27	R, H, K	A, L, I, V, M, Y, F, W
B58	A, S, T	F, W, Y, L, I, V
B62	L, V, M, P, I, Q	F, W, Y, M, I, V

**5 TABLE IV (B): HLA CLASS II SUPERMOTIF**

1	6	9
W, F, Y, V, I, L	A, V, I, L, P, C, S, T	A, V, I, L, C, S, T, M, Y

**Table V: HLA Peptide Scoring Results 55P4H4 – A1, 9-mers**

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	97	STPCGLRG (SEQ ID NO: 21)	112.500
2	15	ISELLDCGY (SEQ ID NO: 22)	67.500
3	29	LSDFDYWDY (SEQ ID NO: 23)	37.500
4	111	NLEIENVCK (SEQ ID NO: 24)	36.000
5	26	ESLLSDFDY (SEQ ID NO: 25)	3.750
6	24	HPESLLSDF (SEQ ID NO: 26)	2.250
7	39	VPEPNLNEV (SEQ ID NO: 27)	2.250
8	133	TFELTLVFK (SEQ ID NO: 28)	1.800
9	170	LSSGFRLVK (SEQ ID NO: 29)	1.500
10	51	ESTCQNLVK (SEQ ID NO: 30)	1.500
11	147	WTSFRDFFF (SEQ ID NO: 31)	1.250

Table V: HLA Peptide Scoring Results 55P4H4 – A1, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
12	125	VCDSSVVPT (SEQ ID NO: 32)	1.000
13	60	MLENCLSKS (SEQ ID NO: 33)	0.900
14	113	EIENVCKKL (SEQ ID NO: 34)	0.900
15	171	SSGFRLVKK (SEQ ID NO: 35)	0.600
16	59	KMLENCLSK (SEQ ID NO: 36)	0.500
17	78	VLVPEKLTQ (SEQ ID NO: 37)	0.500
18	89	AQDVLRLSS (SEQ ID NO: 38)	0.375
19	166	RTLILSSGF (SEQ ID NO: 39)	0.250
20	44	LNEVIFEES (SEQ ID NO: 40)	0.225
21	2	VATGSLSSK (SEQ ID NO: 41)	0.200
22	96	SSTEPGLR (SEQ ID NO: 42)	0.150
23	141	KQENCWTS (SEQ ID NO: 43)	0.135
24	115	ENVCKKLDL (SEQ ID NO: 44)	0.125
25	132	PTFELTLVF (SEQ ID NO: 45)	0.125
26	167	TLILSSGFR (SEQ ID NO: 46)	0.100
27	129	SVVPTFELT (SEQ ID NO: 47)	0.100
28	79	LVPEKLTQR (SEQ ID NO: 48)	0.100
29	37	YVVPEPNLN (SEQ ID NO: 49)	0.100
30	13	ASISELLDC (SEQ ID NO: 50)	0.075
31	128	SSVPTFEL (SEQ ID NO: 51)	0.075
32	75	CSKVLVPEK (SEQ ID NO: 52)	0.060
33	106	CVMHVNLEI (SEQ ID NO: 53)	0.050
34	18	LLDCGYHPE (SEQ ID NO: 54)	0.050
35	168	LILSSGFRL (SEQ ID NO: 55)	0.050
36	120	KLDRIVCDS (SEQ ID NO: 56)	0.050
37	31	DFDYWDYVV (SEQ ID NO: 57)	0.050
38	184	LIGTTVIEG (SEQ ID NO: 58)	0.050
39	130	VVPTFELTL (SEQ ID NO: 59)	0.050
40	41	EPNLNEVIF (SEQ ID NO: 60)	0.050
41	87	RIAQDVLRL (SEQ ID NO: 61)	0.050
42	52	STCQNLVKM (SEQ ID NO: 62)	0.050
43	1	MVATGSLSS (SEQ ID NO: 63)	0.050
44	49	FEESTCQNL (SEQ ID NO: 64)	0.045
45	48	IFEESTCQN (SEQ ID NO: 65)	0.045
46	143	ENCWTSFR (SEQ ID NO: 66)	0.025
47	3	ATGSLSSKN (SEQ ID NO: 67)	0.025
48	174	FRLVKKKLY (SEQ ID NO: 68)	0.025
49	158	GRFSSGFRR (SEQ ID NO: 69)	0.025
50	150	FRDFFFSRG (SEQ ID NO: 70)	0.025

Table VI: HLA Peptide Scoring Results – 55P4H4 – A1, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	97	STEPCLRG (SEQ ID NO: 71)	22.500
2	60	MLENCLSKSK (SEQ ID NO: 72)	18.000
3	111	NLEIENVCKK (SEQ ID NO: 73)	18.000
4	125	VCDSSVPTF (SEQ ID NO: 74)	10.000
5	39	VPEPNLNEVI (SEQ ID NO: 75)	2.250
6	141	KQENCSTWTF (SEQ ID NO: 76)	1.350
7	15	ISELLDCGYH (SEQ ID NO: 77)	1.350
8	78	VLVPEKLTQR (SEQ ID NO: 78)	1.000
9	169	ILSSGFRLVK (SEQ ID NO: 79)	1.000
10	18	LLDCGYHPES (SEQ ID NO: 80)	1.000
11	29	LSDFDYWDYV (SEQ ID NO: 81)	0.750
12	170	LSSGFRLVKK (SEQ ID NO: 82)	0.600
13	129	SVVPTFELTL (SEQ ID NO: 83)	0.500
14	28	LLSDFDYWDY (SEQ ID NO: 84)	0.500
15	14	SISELLDCGY (SEQ ID NO: 85)	0.500
16	171	SSGFRLVKKK (SEQ ID NO: 86)	0.300
17	166	RTLILSSGFR (SEQ ID NO: 87)	0.250
18	150	FRDFFSRGR (SEQ ID NO: 88)	0.250
19	44	LNEVIFEEST (SEQ ID NO: 89)	0.225
20	133	TFELTLVFKQ (SEQ ID NO: 90)	0.225
21	63	NCLSKSKQTK (SEQ ID NO: 91)	0.200
22	1	MVATGSLSSK (SEQ ID NO: 92)	0.200
23	95	LSSTEPCLRL (SEQ ID NO: 93)	0.150
24	147	WTSFRDFFFS (SEQ ID NO: 94)	0.125
25	131	VPTFELTLVF (SEQ ID NO: 95)	0.125
26	110	VNLEIENVCK (SEQ ID NO: 96)	0.100
27	132	PTFELTLVFK (SEQ ID NO: 97)	0.100
28	144	NCSWTSFRDF (SEQ ID NO: 98)	0.100
29	113	EIENVCKKLD (SEQ ID NO: 99)	0.090
30	148	TSFRDFFFSR (SEQ ID NO: 100)	0.075
31	161	SSGFRRTLIL (SEQ ID NO: 101)	0.075
32	96	SSTEPCLRG (SEQ ID NO: 102)	0.075
33	127	DSSVPTFEL (SEQ ID NO: 103)	0.075
34	89	AQDVLRLSST (SEQ ID NO: 104)	0.075
35	183	SLIGTTVIEG (SEQ ID NO: 105)	0.050
36	105	GCVMHVNLEI (SEQ ID NO: 106)	0.050
37	120	KLDRIVCDSS (SEQ ID NO: 107)	0.050
38	37	YVPEPNLNE (SEQ ID NO: 108)	0.050



Table VI: HLA Peptide Scoring Results – 55P4H4 – A1, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
39	50	EESTCQNLVK (SEQ ID NO: 109)	0.050
40	58	VKMLENCLSK (SEQ ID NO: 110)	0.050
41	88	IAQDVLRLSS (SEQ ID NO: 111)	0.050
42	167	TLILSSGFRL (SEQ ID NO: 112)	0.050
43	77	KVLVPEKLTQ (SEQ ID NO: 113)	0.050
44	49	FEESTCQNLV (SEQ ID NO: 114)	0.045
45	48	IFEESTCQNL (SEQ ID NO: 115)	0.045
46	74	GCSKVLVPEK (SEQ ID NO: 116)	0.040
47	51	ESTCQNLVKM (SEQ ID NO: 117)	0.030
48	5	GSLSSKNPAS (SEQ ID NO: 118)	0.030
49	107	VMHVNLEIEN (SEQ ID NO: 119)	0.025
50	136	LTLVFKQENC (SEQ ID NO: 120)	0.025

Table VII: HLA Peptide Scoring Results – 55P4H4 – A2, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	72	KLGCCKVLV (SEQ ID NO: 121)	243.432
2	168	LILSSGFRL (SEQ ID NO: 122)	107.160
3	169	ILSSGFRLV (SEQ ID NO: 123)	44.931
4	180	KLYSLIGTT (SEQ ID NO: 124)	24.955
5	102	GLRGCVMHV (SEQ ID NO: 125)	12.158
6	130	VVPTFELTL (SEQ ID NO: 126)	12.075
7	77	KVLVPEKLT (SEQ ID NO: 127)	8.444
8	87	RIAQDVLRL (SEQ ID NO: 128)	6.756
9	30	SDFDYWDYV (SEQ ID NO: 129)	6.714
10	56	NLVKMLENC (SEQ ID NO: 130)	5.599
11	123	RIVCDSSVV (SEQ ID NO: 131)	3.921
12	106	CVMHVNLEI (SEQ ID NO: 132)	3.378
13	137	TLVFKQENC (SEQ ID NO: 133)	2.434
14	28	LLSDFDYWD (SEQ ID NO: 134)	2.171
15	131	VPTFELTLV (SEQ ID NO: 135)	1.775
16	84	LTQRIAQDV (SEQ ID NO: 136)	1.642
17	27	SLLSDFDYW (SEQ ID NO: 137)	1.412
18	10	KNPASISEL (SEQ ID NO: 138)	1.123
19	140	FKQENC SWT (SEQ ID NO: 139)	1.074
20	116	NVCKKLDRI (SEQ ID NO: 140)	1.029
21	43	NLNEVIFEE (SEQ ID NO: 141)	0.815
22	128	SSVPTFEL (SEQ ID NO: 142)	0.809

Table VII: HLA Peptide Scoring Results – 55P4H4 – A2, 9-mers			
Rank	Start P sition	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
23	129	SVVPTFELT (SEQ ID NO: 143)	0.607
24	59	KMLENCLSK (SEQ ID NO: 144)	0.572
25	160	FSSGFRRTL (SEQ ID NO: 145)	0.488
26	182	YSLIGTTVI (SEQ ID NO: 146)	0.475
27	21	CGYHPESLL (SEQ ID NO: 147)	0.446
28	175	RLVKKKLYS (SEQ ID NO: 148)	0.410
29	110	VNLEIENVC (SEQ ID NO: 149)	0.343
30	53	TCQNLVKML (SEQ ID NO: 150)	0.321
31	179	KKLYSLIGT (SEQ ID NO: 151)	0.308
32	45	NEVIFEEST (SEQ ID NO: 152)	0.270
33	176	LVKKKLYSL (SEQ ID NO: 153)	0.256
34	120	KLDRIVCDS (SEQ ID NO: 154)	0.240
35	95	LSSTEPGL (SEQ ID NO: 155)	0.237
36	109	HVNLEIENV (SEQ ID NO: 156)	0.233
37	162	SGFRRTLIL (SEQ ID NO: 157)	0.212
38	63	NCLSKSKQT (SEQ ID NO: 158)	0.180
39	52	STCQNLVKM (SEQ ID NO: 159)	0.159
40	147	WTSFRDFFF (SEQ ID NO: 160)	0.152
41	104	RGCVMHVNL (SEQ ID NO: 161)	0.139
42	49	FEESTCQNL (SEQ ID NO: 162)	0.122
43	5	GSLSSKNPA (SEQ ID NO: 163)	0.120
44	83	KLTQRIAQD (SEQ ID NO: 164)	0.120
45	7	LSSKNPASI (SEQ ID NO: 165)	0.116
46	39	VPEPNLNEV (SEQ ID NO: 166)	0.114
47	57	LVKMLENCL (SEQ ID NO: 167)	0.111
48	148	TSFRDFFFS (SEQ ID NO: 168)	0.109
49	50	EESTCQNLV (SEQ ID NO: 169)	0.101
50	125	VCDSSVVPT (SEQ ID NO: 170)	0.076

Table VIII: HLA Peptide Scoring Results – 55P4H4 – A2, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	180	KLYSLIGTTV (SEQ ID NO: 171)	778.982
2	83	KLTQRIAQDV (SEQ ID NO: 172)	243.432
3	167	TLILSSGFRL (SEQ ID NO: 173)	123.902
4	38	VVPEPNLNEV (SEQ ID NO: 174)	97.561
5	175	RLVKKKLYSL (SEQ ID NO: 175)	49.134
6	69	KQTKLGCSKV (SEQ ID NO: 176)	24.681

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Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
7	130	VVPTFELTLV (SEQ ID NO: 177)	23.795
8	168	LILSSGFRLV (SEQ ID NO: 178)	22.858
9	94	RLSSTEPCLG (SEQ ID NO: 179)	21.362
10	56	NLVKMLENCL (SEQ ID NO: 180)	21.362
11	64	CLSKSKQTKL (SEQ ID NO: 181)	21.362
12	124	IVCDSSVVPT (SEQ ID NO: 182)	10.453
13	6	SLSSKNPASI (SEQ ID NO: 183)	10.433
14	129	SVVPTFELTL (SEQ ID NO: 184)	7.103
15	59	KMLENCLSKS (SEQ ID NO: 185)	6.572
16	79	LVPEKLTQRI (SEQ ID NO: 186)	6.363
17	28	LLSDFDYWDY (SEQ ID NO: 187)	5.843
18	101	CGLRGCVMHV (SEQ ID NO: 188)	3.864
19	112	LEIENVCKKL (SEQ ID NO: 189)	2.895
20	29	LSDFDYWDYV (SEQ ID NO: 190)	1.414
21	27	SLLSDFDYWD (SEQ ID NO: 191)	1.153
22	89	AQDVLRLSST (SEQ ID NO: 192)	0.695
23	52	STCQNLVKML (SEQ ID NO: 193)	0.682
24	30	SDFDYWDYVV (SEQ ID NO: 194)	0.601
25	172	SGFRLVKKKL (SEQ ID NO: 195)	0.516
26	116	NVCKKLDRIV (SEQ ID NO: 196)	0.499
27	35	WDYVVPEPNL (SEQ ID NO: 197)	0.437
28	49	FEESTCQNLV (SEQ ID NO: 198)	0.398
29	71	TKLGCSKVLV (SEQ ID NO: 199)	0.357
30	92	VLRLSSTEPCL (SEQ ID NO: 200)	0.315
31	160	FSSGFRRTLI (SEQ ID NO: 201)	0.313
32	147	WTSFRDFFFS (SEQ ID NO: 202)	0.289
33	43	NLNEVIFEEES (SEQ ID NO: 203)	0.284
34	47	VIFEESTCQN (SEQ ID NO: 204)	0.264
35	10	KNPASISELL (SEQ ID NO: 205)	0.239
36	136	LTLVFKQENC (SEQ ID NO: 206)	0.213
37	98	TEPCGLRGCV (SEQ ID NO: 207)	0.176
38	183	SLIGTTVIEG (SEQ ID NO: 208)	0.171
39	55	QNLVKMLENC (SEQ ID NO: 209)	0.135
40	84	LTQRIAQDVL (SEQ ID NO: 210)	0.101
41	45	NEVIFEESTC (SEQ ID NO: 211)	0.097
42	108	MHVNLEIENV (SEQ ID NO: 212)	0.093
43	107	VMHVNLEIEN (SEQ ID NO: 213)	0.091
44	176	LVKKKLYSLI (SEQ ID NO: 214)	0.081
45	137	TLVFKQENCS (SEQ ID NO: 215)	0.075

Table VIII: HLA Peptide Scoring Results – 55P4H4 – A2, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
46	9	SKNPASISEL (SEQ ID NO: 216)	0.068
47	105	GCVMHVNLEI (SEQ ID NO: 217)	0.068
48	127	DSSVVPTFEL (SEQ ID NO: 218)	0.061
49	78	VLVPEKLTQR (SEQ ID NO: 219)	0.058
50	161	SSGFRRTLIL (SEQ ID NO: 220)	0.057

Table IX: HLA Peptide Scoring Results – 55P4H4 – A3, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	59	KMLENCLSK (SEQ ID NO: 221)	270.000
2	64	CLSKSKQTK (SEQ ID NO: 222)	20.000
3	111	NLEIENVCK (SEQ ID NO: 223)	20.000
4	102	GLRGCVMHV (SEQ ID NO: 224)	5.400
5	167	TLILSSGFR (SEQ ID NO: 225)	1.800
6	69	KQTKLGCSK (SEQ ID NO: 226)	1.800
7	79	LVPEKLTQR (SEQ ID NO: 227)	0.900
8	170	LSSGFRLVK (SEQ ID NO: 228)	0.900
9	27	SLLSDFDYW (SEQ ID NO: 229)	0.900
10	180	KLYSLIGTT (SEQ ID NO: 230)	0.675
11	72	KLGCCKVLV (SEQ ID NO: 231)	0.600
12	120	KLDRIVCDS (SEQ ID NO: 232)	0.540
13	56	NLVKMLENC (SEQ ID NO: 233)	0.450
14	2	VATGSLSSK (SEQ ID NO: 234)	0.450
15	112	LEIENVCKK (SEQ ID NO: 235)	0.405
16	87	RIAQDVLRL (SEQ ID NO: 236)	0.360
17	130	VVPTFELTL (SEQ ID NO: 237)	0.360
18	75	CSKVLVPEK (SEQ ID NO: 238)	0.300
19	147	WTSFRDFFF (SEQ ID NO: 239)	0.300
20	137	TLVFKQENC (SEQ ID NO: 240)	0.300
21	176	LVKKKLYSL (SEQ ID NO: 241)	0.270
22	168	LILSSGFRL (SEQ ID NO: 242)	0.270
23	106	CVMHVNLEI (SEQ ID NO: 243)	0.270
24	43	NLNEVIFEE (SEQ ID NO: 244)	0.203
25	158	GRFSSGFRR (SEQ ID NO: 245)	0.180
26	28	LLSDFDYWD (SEQ ID NO: 246)	0.180
27	132	PTFELTLVF (SEQ ID NO: 247)	0.150
28	172	SGFRLVKKK (SEQ ID NO: 248)	0.150
29	171	SSGFRLVKK (SEQ ID NO: 249)	0.150



<b>Rank</b>	<b>Start Position</b>	<b>Subsequence Residue Listing</b>	<b>Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)</b>
14	56	NLVKMLENCL (SEQ ID NO: 284)	0.900
15	129	SVVPTFELTL (SEQ ID NO: 285)	0.810
16	6	SLSSKNPASI (SEQ ID NO: 286)	0.600
17	14	SISELLDCGY (SEQ ID NO: 287)	0.600
18	64	CLSKSKQTKL (SEQ ID NO: 288)	0.600
19	94	RLSSTEPCLG (SEQ ID NO: 289)	0.600
20	59	KMLENCLSKS (SEQ ID NO: 290)	0.405
21	72	KLGC SKVLVP (SEQ ID NO: 291)	0.360
22	63	NCLSKSKQTK (SEQ ID NO: 292)	0.300
23	43	NLNEVIFEES (SEQ ID NO: 293)	0.270
24	27	SLLSDFDYWD (SEQ ID NO: 294)	0.270
25	183	SLIGTTVIEG (SEQ ID NO: 295)	0.270
26	170	LSSGFRLVKK (SEQ ID NO: 296)	0.225
27	102	GLRGCVMHVN (SEQ ID NO: 297)	0.203
28	92	VLRLSSTEPCL (SEQ ID NO: 298)	0.200
29	141	KQENC SWTSF (SEQ ID NO: 299)	0.180
30	120	KLDRIVCDSS (SEQ ID NO: 300)	0.180
31	171	SSGFRLVKKK (SEQ ID NO: 301)	0.150
32	145	CSWTSFRDFF (SEQ ID NO: 302)	0.150
33	18	LLDCGYHPES (SEQ ID NO: 303)	0.120
34	85	TQRIAQDVLRL (SEQ ID NO: 304)	0.120
35	138	LVFKQENC SW (SEQ ID NO: 305)	0.100
36	110	VNLEIENVCK (SEQ ID NO: 306)	0.090
37	125	VCDSSVVPTF (SEQ ID NO: 307)	0.090
38	166	RTLILSSGFR (SEQ ID NO: 308)	0.090
39	79	LVPEKLTQRI (SEQ ID NO: 309)	0.090
40	105	GCVMHVNLEI (SEQ ID NO: 310)	0.081
41	58	VKMLENCLSK (SEQ ID NO: 311)	0.060
42	176	LVKKKLYSLI (SEQ ID NO: 312)	0.060
43	137	TLVFKQENC S (SEQ ID NO: 313)	0.060
44	38	VVPEPNLNEV (SEQ ID NO: 314)	0.045
45	155	FSRGRFSSGF (SEQ ID NO: 315)	0.045
46	52	STCQNLVKML (SEQ ID NO: 316)	0.045
47	107	VMHVNLEIEN (SEQ ID NO: 317)	0.040
48	131	VPTFELTLVF (SEQ ID NO: 318)	0.040
49	142	QENC SWTSFR (SEQ ID NO: 319)	0.036
50	50	EESTCQNLVK (SEQ ID NO: 320)	0.036

Table XI: HLA Peptide Scoring Results – 55P4H4 – A11, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	59	KMLENCLSK (SEQ ID NO: 321)	3.600
2	69	KQTKLGCSK (SEQ ID NO: 322)	1.800
3	64	CLSKSKQTK (SEQ ID NO: 323)	0.400
4	79	LVPEKLTQR (SEQ ID NO: 324)	0.400
5	111	NLEIENVCK (SEQ ID NO: 325)	0.400
6	2	VATGSLSSK (SEQ ID NO: 326)	0.200
7	133	TFELTLVFK (SEQ ID NO: 327)	0.200
8	149	SFRDFFFSR (SEQ ID NO: 328)	0.120
9	167	TLILSSGFR (SEQ ID NO: 329)	0.120
10	112	LEIENVCKK (SEQ ID NO: 330)	0.090
11	106	CVMHVNLEI (SEQ ID NO: 331)	0.080
12	158	GRFSSGFRR (SEQ ID NO: 332)	0.072
13	166	RTLILSSGF (SEQ ID NO: 333)	0.045
14	176	LVKKKLYSL (SEQ ID NO: 334)	0.040
15	130	VVPTFELTL (SEQ ID NO: 335)	0.040
16	170	LSSGFRLVK (SEQ ID NO: 336)	0.040
17	61	LENCLSKSK (SEQ ID NO: 337)	0.030
18	147	WTSFRDFFF (SEQ ID NO: 338)	0.030
19	87	RIAQDVLRL (SEQ ID NO: 339)	0.024
20	102	GLRGCVMHV (SEQ ID NO: 340)	0.024
21	116	NVCKKLDRI (SEQ ID NO: 341)	0.020
22	75	CSKVLVPEK (SEQ ID NO: 342)	0.020
23	57	LVKMLENCL (SEQ ID NO: 343)	0.020
24	171	SSGFRLVKK (SEQ ID NO: 344)	0.020
25	109	HVNLEIENV (SEQ ID NO: 345)	0.020
26	172	SGFRLVKKK (SEQ ID NO: 346)	0.020
27	123	RIVCDSSVV (SEQ ID NO: 347)	0.018
28	168	LILSSGFRL (SEQ ID NO: 348)	0.018
29	157	RGRFSSGFR (SEQ ID NO: 349)	0.012
30	72	KLGCCKVLV (SEQ ID NO: 350)	0.012
31	51	ESTCQNLVK (SEQ ID NO: 351)	0.012
32	70	QTKLGCSKV (SEQ ID NO: 352)	0.010
33	84	LTQRIAQDV (SEQ ID NO: 353)	0.010
34	52	STCQNLVKM (SEQ ID NO: 354)	0.010
35	115	ENVCKKLDL (SEQ ID NO: 355)	0.007
36	86	QRIAQDVL (SEQ ID NO: 356)	0.006
37	85	TQRIAQDVL (SEQ ID NO: 357)	0.006
38	27	SLLSDFDYW (SEQ ID NO: 358)	0.006
39	77	KVLVPEKLT (SEQ ID NO: 359)	0.005

Table XI: HLA Peptide Scoring Results – 55P4H4 – A11, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
40	96	SSTEPCLGR (SEQ ID NO: 360)	0.004
41	38	VVPEPNLNE (SEQ ID NO: 361)	0.004
42	1	MVATGSLSS (SEQ ID NO: 362)	0.004
43	181	LYSLIGTTV (SEQ ID NO: 363)	0.004
44	132	PTFELTLVF (SEQ ID NO: 364)	0.004
45	138	LVFKQENCSS (SEQ ID NO: 365)	0.004
46	141	KQENCSSWTS (SEQ ID NO: 366)	0.004
47	175	RLVKKKLYS (SEQ ID NO: 367)	0.004
48	37	YVVPEPNLN (SEQ ID NO: 368)	0.003
49	173	GFRLVKKKL (SEQ ID NO: 369)	0.003
50	129	SVVPTFELT (SEQ ID NO: 370)	0.003

Table XII: HLA Peptide Scoring Results – 55P4H4 – A11, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	1	MVATGSLSSK (SEQ ID NO: 371)	2.000
2	166	RTLILSSGFR (SEQ ID NO: 372)	0.900
3	169	ILSSGFRLVK (SEQ ID NO: 373)	0.800
4	74	GCSKVLVPEK (SEQ ID NO: 374)	0.600
5	111	NLEIENVCKK (SEQ ID NO: 375)	0.400
6	63	NCLSKSKQTK (SEQ ID NO: 376)	0.300
7	60	MLENCLSKSK (SEQ ID NO: 377)	0.200
8	132	PTFELTLVFK (SEQ ID NO: 378)	0.200
9	78	VLVPEKLTQR (SEQ ID NO: 379)	0.120
10	85	TQRIAQDVLR (SEQ ID NO: 380)	0.120
11	58	VKMLENCLSK (SEQ ID NO: 381)	0.080
12	110	VNLEIENVCK (SEQ ID NO: 382)	0.060
13	129	SVVPTFELTL (SEQ ID NO: 383)	0.060
14	138	LVFKQENCSSW (SEQ ID NO: 384)	0.040
15	157	RGRFSSGFRR (SEQ ID NO: 385)	0.036
16	50	EESTCQNLVK (SEQ ID NO: 386)	0.036
17	175	RLVKKKLYSL (SEQ ID NO: 387)	0.036
18	148	TSFRDFFFSR (SEQ ID NO: 388)	0.024
19	180	KLYSLIGTTV (SEQ ID NO: 389)	0.024
20	114	IENVCKKLDL (SEQ ID NO: 390)	0.024
21	130	VVPTFELTLV (SEQ ID NO: 391)	0.020
22	176	LVKKKLYSLI (SEQ ID NO: 392)	0.020
23	68	SKQTKLGCSK (SEQ ID NO: 393)	0.020



Table XII: HLA Peptide Scoring Results – 55P4H4 – A11, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
24	38	VVPEPNLNEV (SEQ ID NO: 394)	0.020
25	170	LSSGFRLVKK (SEQ ID NO: 395)	0.020
26	79	LVPEKLTQRI (SEQ ID NO: 396)	0.020
27	141	KQENCWSWTSF (SEQ ID NO: 397)	0.018
28	77	KVLVPEKLTQ (SEQ ID NO: 398)	0.018
29	69	KQTKLGCSKV (SEQ ID NO: 399)	0.018
30	167	TLILSSGFRL (SEQ ID NO: 400)	0.018
31	105	GCVMHVNLEI (SEQ ID NO: 401)	0.018
32	94	RLSSTEPCLG (SEQ ID NO: 402)	0.012
33	142	QENCWSWTSFR (SEQ ID NO: 403)	0.012
34	83	KLTQRIAQDV (SEQ ID NO: 404)	0.012
35	171	SSGFRLVKKK (SEQ ID NO: 405)	0.010
36	84	LTQRIAQDVL (SEQ ID NO: 406)	0.010
37	28	LLSDFDYWDY (SEQ ID NO: 407)	0.008
38	56	NLVKMLENCL (SEQ ID NO: 408)	0.006
39	37	YVVPEPNLNE (SEQ ID NO: 409)	0.006
40	52	STCQNLVKML (SEQ ID NO: 410)	0.005
41	70	QTKLGCSKVL (SEQ ID NO: 411)	0.005
42	106	CVMHVNLEIE (SEQ ID NO: 412)	0.004
43	156	SRGRFSSGFR (SEQ ID NO: 413)	0.004
44	95	LSSTEPCLGR (SEQ ID NO: 414)	0.004
45	131	VPTFELTLVF (SEQ ID NO: 415)	0.004
46	64	CLS KSKQTKL (SEQ ID NO: 416)	0.004
47	181	LYSLIGTTVI (SEQ ID NO: 417)	0.004
48	14	SISELLDCGY (SEQ ID NO: 418)	0.004
49	6	SLSSKNPASI (SEQ ID NO: 419)	0.004
50	173	GFRLVKKKLY (SEQ ID NO: 420)	0.003

Table XIII: HLA Peptide Scoring Results – 55P4H4 – A24, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	36	DYVVPEPNL (SEQ ID NO: 421)	300.000
2	173	GFRLVKKKL (SEQ ID NO: 422)	30.800
3	10	KNPASISEL (SEQ ID NO: 423)	13.200
4	152	DFFFSRGRF (SEQ ID NO: 424)	10.000
5	113	EIENVCKKL (SEQ ID NO: 425)	9.240
6	87	RIAQDVLRL (SEQ ID NO: 426)	8.000
7	104	RGCVMHVN (SEQ ID NO: 427)	8.000

Table XIII: HLA Peptide Scoring Results – 55P4H4 – A24, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
8	53	TCQNLVKML (SEQ ID NO: 428)	7.200
9	166	RTLILSSGF (SEQ ID NO: 429)	7.200
10	181	LYSLIGTTV (SEQ ID NO: 430)	7.000
11	128	SSVPTFEL (SEQ ID NO: 431)	6.600
12	168	LILSSGFRL (SEQ ID NO: 432)	6.000
13	130	VVPTFELTL (SEQ ID NO: 433)	6.000
14	22	GYHPESLLS (SEQ ID NO: 434)	6.000
15	57	LVKMLENCL (SEQ ID NO: 435)	5.760
16	11	NPASISELL (SEQ ID NO: 436)	5.600
17	65	LSKSKQTKL (SEQ ID NO: 437)	4.400
18	20	DCGYHPESL (SEQ ID NO: 438)	4.000
19	21	CGYHPESLL (SEQ ID NO: 439)	4.000
20	176	LVKKKLYSL (SEQ ID NO: 440)	4.000
21	162	SGFRRTLIL (SEQ ID NO: 441)	4.000
22	160	FSSGFRRTL (SEQ ID NO: 442)	4.000
23	85	TQRIAQDVL (SEQ ID NO: 443)	4.000
24	95	LSSTEPCL (SEQ ID NO: 444)	4.000
25	24	HPESLLSDF (SEQ ID NO: 445)	3.600
26	41	EPNLNEVIF (SEQ ID NO: 446)	3.000
27	145	CSWTSFRDF (SEQ ID NO: 447)	2.400
28	106	CVMHVNLEI (SEQ ID NO: 448)	2.310
29	80	VPEKLTQRI (SEQ ID NO: 449)	2.160
30	147	WTSFRDFFF (SEQ ID NO: 450)	2.000
31	146	SWTSFRDFF (SEQ ID NO: 451)	2.000
32	182	YSLIGTTVI (SEQ ID NO: 452)	1.500
33	159	RFSSGFRRT (SEQ ID NO: 453)	1.200
34	7	LSSKNPASI (SEQ ID NO: 454)	1.000
35	161	SSGFRRTLI (SEQ ID NO: 455)	1.000
36	116	NVCKKLDRI (SEQ ID NO: 456)	1.000
37	76	SKVLVPEKL (SEQ ID NO: 457)	0.924
38	48	IFEESTCQN (SEQ ID NO: 458)	0.900
39	49	FEESTCQNL (SEQ ID NO: 459)	0.720
40	71	TKLGCSKVL (SEQ ID NO: 460)	0.600
41	33	DYWDYVVPE (SEQ ID NO: 461)	0.600
42	52	STCQNLVKM (SEQ ID NO: 462)	0.550
43	163	GFRRTLILS (SEQ ID NO: 463)	0.500
44	153	FFFSRGRFS (SEQ ID NO: 464)	0.500
45	154	FFSRGRFSS (SEQ ID NO: 465)	0.500
46	139	VFKQENCWS (SEQ ID NO: 466)	0.500

Table XIII: HLA Peptide Scoring Results – 55P4H4 – A24, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
47	31	DFDYWDYVV (SEQ ID NO: 467)	0.500
48	110	VNLEIENVC (SEQ ID NO: 468)	0.302
49	175	RLVKKKLYS (SEQ ID NO: 469)	0.300
50	77	KVLVPEKLT (SEQ ID NO: 470)	0.300

Table XIV: HLA Peptide Scoring Results – 55P4H4 – A24, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	181	LYSLIGTTVI (SEQ ID NO: 471)	50.000
2	48	IFEESTCQNL (SEQ ID NO: 472)	43.200
3	159	RFSSGFRRTL (SEQ ID NO: 473)	40.000
4	10	KNPASISELL (SEQ ID NO: 474)	16.800
5	175	RLVKKKLYSL (SEQ ID NO: 475)	12.000
6	36	DYVVPEPNLN (SEQ ID NO: 476)	9.000
7	56	NLVKMLENCL (SEQ ID NO: 477)	8.640
8	94	RLSSTEPCL (SEQ ID NO: 478)	8.000
9	129	SVVPTFELTL (SEQ ID NO: 479)	7.200
10	75	CSKVLVPEKL (SEQ ID NO: 480)	6.160
11	172	SGFRLVKKKL (SEQ ID NO: 481)	6.160
12	141	KQENCSTWTSF (SEQ ID NO: 482)	6.000
13	167	TLILSSGFRL (SEQ ID NO: 483)	6.000
14	84	LTQRIAQDVL (SEQ ID NO: 484)	6.000
15	52	STCQNLVKML (SEQ ID NO: 485)	4.800
16	127	DSSVVPTFEL (SEQ ID NO: 486)	4.400
17	64	CLSKSKQTKL (SEQ ID NO: 487)	4.400
18	161	SSGFRRTLIL (SEQ ID NO: 488)	4.000
19	20	DCGYHPESLL (SEQ ID NO: 489)	4.000
20	70	QTKLGCSKVL (SEQ ID NO: 490)	4.000
21	125	VCDSSVVPTF (SEQ ID NO: 491)	2.800
22	79	LVPEKLTQRI (SEQ ID NO: 492)	2.592
23	144	NCSWTSFRDF (SEQ ID NO: 493)	2.400
24	131	VPTFELTLVF (SEQ ID NO: 494)	2.400
25	105	GCVMHVNLEI (SEQ ID NO: 495)	2.310
26	39	VPEPNLNEVI (SEQ ID NO: 496)	2.160
27	146	SWTSFRDFFF (SEQ ID NO: 497)	2.000
28	145	CSWTSFRDFF (SEQ ID NO: 498)	2.000
29	155	FSRGRFSSGF (SEQ ID NO: 499)	2.000
30	115	ENVCKKLDRI (SEQ ID NO: 500)	1.500



Table XV: HLA Peptide Scoring Results – 55P4H4 – B7, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	168	LILSSGFRL (SEQ ID NO: 535)	4.000
16	131	VPTFELTLV (SEQ ID NO: 536)	4.000
17	95	LSSTEPCLL (SEQ ID NO: 537)	4.000
18	162	SGFRRTLIL (SEQ ID NO: 538)	4.000
19	10	KNPASISEL (SEQ ID NO: 539)	4.000
20	20	DCGYHPESL (SEQ ID NO: 540)	4.000
21	104	RGCVMHVN (SEQ ID NO: 541)	4.000
22	80	VPEKLTQRI (SEQ ID NO: 542)	2.000
23	102	GLRGCVMHV (SEQ ID NO: 543)	2.000
24	116	NVCKKLDRI (SEQ ID NO: 544)	2.000
25	113	EIENVCKKL (SEQ ID NO: 545)	1.200
26	39	VPEPNLNEV (SEQ ID NO: 546)	1.200
27	52	STCQNLVKM (SEQ ID NO: 547)	1.000
28	109	HVNLEIENV (SEQ ID NO: 548)	1.000
29	77	KVLVPEKLT (SEQ ID NO: 549)	0.750
30	161	SSGFRRTLI (SEQ ID NO: 550)	0.600
31	36	DYVVPEPNL (SEQ ID NO: 551)	0.600
32	46	EVIFEESTC (SEQ ID NO: 552)	0.500
33	129	SVVPTFELT (SEQ ID NO: 553)	0.500
34	71	TKLGCSKVL (SEQ ID NO: 554)	0.400
35	7	LSSKNPASI (SEQ ID NO: 555)	0.400
36	182	YSLIGTTVI (SEQ ID NO: 556)	0.400
37	41	EPNLNEVIF (SEQ ID NO: 557)	0.400
38	76	SKVLVPEKL (SEQ ID NO: 558)	0.400
39	13	ASISELLDC (SEQ ID NO: 559)	0.300
40	84	LTQRIAQDV (SEQ ID NO: 560)	0.200
41	72	KLGCCKVLV (SEQ ID NO: 561)	0.200
42	169	ILSSGFRLV (SEQ ID NO: 562)	0.200
43	123	RIVCDSSVV (SEQ ID NO: 563)	0.200
44	117	VCKKLDRIV (SEQ ID NO: 564)	0.200
45	70	QTKLGCSKV (SEQ ID NO: 565)	0.200
46	24	HPESLLSDF (SEQ ID NO: 566)	0.120
47	49	FEESTCQNL (SEQ ID NO: 567)	0.120
48	37	YVVPEPNLN (SEQ ID NO: 568)	0.100
49	155	FSRGRFSSG (SEQ ID NO: 569)	0.100
50	1	MVATGSLSS (SEQ ID NO: 570)	0.100

Table XVI: HLA Peptide Scoring Results – 55P4H4 – B7, 10-mers			
Rank	Start	Subsequence Residue Listing	Score (Estimate of Half Time of

	Position		Disassociation of a Molecule Containing This Subsequence)
1	129	SVVPTFELTL (SEQ ID NO: 571)	20.000
2	99	EPCGLRGCVL (SEQ ID NO: 572)	20.000
3	20	DCGYHPESLL (SEQ ID NO: 573)	6.000
4	127	DSSVVPTFEL (SEQ ID NO: 574)	6.000
5	167	TLILSSGFRL (SEQ ID NO: 575)	4.000
6	172	SGFRLVKKKL (SEQ ID NO: 576)	4.000
7	70	QTKLGCSKVL (SEQ ID NO: 577)	4.000
8	94	RLSSTEPCL (SEQ ID NO: 578)	4.000
9	161	SSGFRRTLIL (SEQ ID NO: 579)	4.000
10	75	CSKVLVPEKL (SEQ ID NO: 580)	4.000
11	175	RLVKKKLYSL (SEQ ID NO: 581)	4.000
12	52	STCQNLVKML (SEQ ID NO: 582)	4.000
13	10	KNPASISELL (SEQ ID NO: 583)	4.000
14	64	CLSKSKQTKL (SEQ ID NO: 584)	4.000
15	84	LTQRIAQDVL (SEQ ID NO: 585)	4.000
16	56	NLVKMLENCL (SEQ ID NO: 586)	4.000
17	39	VPEPNLNEVI (SEQ ID NO: 587)	2.400
18	176	LVKKKLYSLI (SEQ ID NO: 588)	2.000
19	79	LVPEKLTQRI (SEQ ID NO: 589)	2.000
20	116	NVCKKLDRIV (SEQ ID NO: 590)	1.000
21	92	VLRLSSTEPCL (SEQ ID NO: 591)	1.000
22	51	ESTCQNLVKM (SEQ ID NO: 592)	1.000
23	130	VVPTFELTLV (SEQ ID NO: 593)	1.000
24	38	VVPEPNLNEV (SEQ ID NO: 594)	1.000
25	35	WDYVVPEPNL (SEQ ID NO: 595)	0.600
26	160	FSSGFRRTLI (SEQ ID NO: 596)	0.600
27	80	VPEKLTQRIA (SEQ ID NO: 597)	0.600
28	159	RFSSGFRRTL (SEQ ID NO: 598)	0.600
29	124	IVCDSSVVPT (SEQ ID NO: 599)	0.500
30	109	HVNLEIENVCL (SEQ ID NO: 600)	0.500
31	9	SKNPASISEL (SEQ ID NO: 601)	0.400
32	131	VPTFELTLVF (SEQ ID NO: 602)	0.400
33	19	LDCGYHPESL (SEQ ID NO: 603)	0.400
34	6	SLSSKNPASI (SEQ ID NO: 604)	0.400
35	103	LRGCVLMHVN (SEQ ID NO: 605)	0.400
36	105	GCVLMHVNLEI (SEQ ID NO: 606)	0.400
37	112	LEIENVCKKL (SEQ ID NO: 607)	0.400
38	86	QRIAQDVLRL (SEQ ID NO: 608)	0.400
39	115	ENVCKKLDRI (SEQ ID NO: 609)	0.400
40	155	FSRGRFSSGF (SEQ ID NO: 610)	0.200
41	180	KLYSLIGTTV (SEQ ID NO: 611)	0.200

Table XVI: HLA Peptide Scoring Results – 55P4H4 – B7, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
42	41	EPNLNEVIFE (SEQ ID NO: 612)	0.200
43	11	NPASISELLD (SEQ ID NO: 613)	0.200
44	101	CGLRGCVMHV (SEQ ID NO: 614)	0.200
45	102	GLRGCVMHVN (SEQ ID NO: 615)	0.200
46	69	KQTKLGCSKV (SEQ ID NO: 616)	0.200
47	121	LDRIVCDSSV (SEQ ID NO: 617)	0.200
48	83	KLTQRIAQDV (SEQ ID NO: 618)	0.200
49	168	LILSSGFRLV (SEQ ID NO: 619)	0.200
50	117	VCKKLDRIVC (SEQ ID NO: 620)	0.150

Table XVII: HLA Peptide Scoring Results – 55P4H4 – B35, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	41	EPNLNEVIF (SEQ ID NO: 621)	20.000
2	11	NPASISELL (SEQ ID NO: 622)	20.000
3	65	LSKSKQTKL (SEQ ID NO: 623)	15.000
4	26	ESLLSDFDY (SEQ ID NO: 624)	10.000
5	95	LSSTEPCL (SEQ ID NO: 625)	7.500
6	131	VPTFELTLV (SEQ ID NO: 626)	6.000
7	24	HPESLLSDF (SEQ ID NO: 627)	6.000
8	128	SSVPTFEL (SEQ ID NO: 628)	5.000
9	145	CSWTSFRDF (SEQ ID NO: 629)	5.000
10	160	FSSGFRRTL (SEQ ID NO: 630)	5.000
11	29	LSDFDYWDY (SEQ ID NO: 631)	4.500
12	99	EPCGLRGCV (SEQ ID NO: 632)	4.000
13	87	RIAQDVLRL (SEQ ID NO: 633)	3.000
14	85	TQRIAQDVL (SEQ ID NO: 634)	3.000
15	176	LVKKKLYSL (SEQ ID NO: 635)	3.000
16	15	ISELLDCGY (SEQ ID NO: 636)	3.000
17	67	KSKQTKLGC (SEQ ID NO: 637)	3.000
18	57	LVKMLENCL (SEQ ID NO: 638)	3.000
19	80	VPEKLTQRI (SEQ ID NO: 639)	2.400
20	161	SSGFRRTLI (SEQ ID NO: 640)	2.000
21	7	LSSKNPASI (SEQ ID NO: 641)	2.000
22	182	YSLIGTTVI (SEQ ID NO: 642)	2.000
23	52	STCQNLVKM (SEQ ID NO: 643)	2.000
24	104	RGCVMHVN (SEQ ID NO: 644)	2.000
25	10	KNPASISEL (SEQ ID NO: 645)	2.000

Table XVII: HLA Peptide Scoring Results – 55P4H4 – B35, 9-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
26	166	RTLILSSGF (SEQ ID NO: 646)	2.000
27	8	SSKNPASIS (SEQ ID NO: 647)	1.500
28	39	VPEPNLNEV (SEQ ID NO: 648)	1.200
29	168	LILSSGFRL (SEQ ID NO: 649)	1.000
30	162	SGFRRTLIL (SEQ ID NO: 650)	1.000
31	53	TCQNLVKML (SEQ ID NO: 651)	1.000
32	20	DCGYHPESL (SEQ ID NO: 652)	1.000
33	130	VVPTFELTL (SEQ ID NO: 653)	1.000
34	21	CGYHPESLL (SEQ ID NO: 654)	1.000
35	147	WTSFRDFFF (SEQ ID NO: 655)	1.000
36	27	SLLSDFDYW (SEQ ID NO: 656)	0.750
37	13	ASISELLDC (SEQ ID NO: 657)	0.750
38	148	TSFRDFFFS (SEQ ID NO: 658)	0.750
39	117	VCKKLDRIV (SEQ ID NO: 659)	0.600
40	88	IAQDVLRLS (SEQ ID NO: 660)	0.600
41	123	RIVCDSSVV (SEQ ID NO: 661)	0.600
42	70	QTKLGCSKV (SEQ ID NO: 662)	0.600
43	102	GLRGCVMHV (SEQ ID NO: 663)	0.600
44	5	GSLSSKNPA (SEQ ID NO: 664)	0.500
45	72	KLGCSKVLV (SEQ ID NO: 665)	0.400
46	116	NVCKKLDRI (SEQ ID NO: 666)	0.400
47	106	CVMHVNLEI (SEQ ID NO: 667)	0.400
48	113	EIENVCKKL (SEQ ID NO: 668)	0.300
49	109	HVNLEIENV (SEQ ID NO: 669)	0.300
50	173	GFRLVKKKL (SEQ ID NO: 670)	0.300

Table XVIII: HLA Peptide Scoring Results – 55P4H4 – B35, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	99	EPCGLRGCV (SEQ ID NO: 671)	40.000
2	131	VPTFELTLVF (SEQ ID NO: 672)	20.000
3	75	CSKVLVPEKL (SEQ ID NO: 673)	15.000
4	155	FSRGRFSSGF (SEQ ID NO: 674)	15.000
5	51	ESTCQNLVKM (SEQ ID NO: 675)	10.000
6	28	LLSDFDYWDY (SEQ ID NO: 676)	6.000
7	161	SSGFRRTLIL (SEQ ID NO: 677)	5.000
8	145	CSWTSFRDFF (SEQ ID NO: 678)	5.000
9	127	DSSVVPTFEL (SEQ ID NO: 679)	5.000



Table XVIII: HLA Peptide Scoring Results – 55P4H4 – B35, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
10	14	SISELLDCGY (SEQ ID NO: 680)	4.000
11	26	ESLLSDFDYW (SEQ ID NO: 681)	3.750
12	94	RLSSTEPCLG (SEQ ID NO: 682)	3.000
13	67	KSKQTKLGCS (SEQ ID NO: 683)	3.000
14	70	QTKLGCSKVL (SEQ ID NO: 684)	3.000
15	39	VPEPNLNEVI (SEQ ID NO: 685)	2.400
16	160	FSSGFRRTL (SEQ ID NO: 686)	2.000
17	175	RLVKKKLYSL (SEQ ID NO: 687)	2.000
18	10	KNPASISELL (SEQ ID NO: 688)	2.000
19	176	LVKKKLYSLI (SEQ ID NO: 689)	1.200
20	56	NLVKMLENCL (SEQ ID NO: 690)	1.000
21	167	TLILSSGFRL (SEQ ID NO: 691)	1.000
22	129	SVVPTFELTL (SEQ ID NO: 692)	1.000
23	20	DCGYHPESLL (SEQ ID NO: 693)	1.000
24	144	NCSWTSFRDF (SEQ ID NO: 694)	1.000
25	84	LTQRIAQDVL (SEQ ID NO: 695)	1.000
26	172	SGFRLVKKKL (SEQ ID NO: 696)	1.000
27	52	STCQNLVKML (SEQ ID NO: 697)	1.000
28	64	CLS KSKQTKL (SEQ ID NO: 698)	1.000
29	79	LVPEKLTQRI (SEQ ID NO: 699)	0.800
30	138	LVFKQENCSW (SEQ ID NO: 700)	0.750
31	88	IAQDVLRLSS (SEQ ID NO: 701)	0.600
32	80	VPEKLTQRIA (SEQ ID NO: 702)	0.600
33	173	GFRLVKKKLY (SEQ ID NO: 703)	0.600
34	141	KQENCSWTSF (SEQ ID NO: 704)	0.600
35	5	GSLSSKNPAS (SEQ ID NO: 705)	0.500
36	128	SSVPTFELT (SEQ ID NO: 706)	0.500
37	7	LSSKNPASIS (SEQ ID NO: 707)	0.500
38	57	LVKMLENCLS (SEQ ID NO: 708)	0.450
39	117	VCKKLDIVC (SEQ ID NO: 709)	0.450
40	105	GCVMHVNLEI (SEQ ID NO: 710)	0.400
41	59	KMLENCLSKS (SEQ ID NO: 711)	0.400
42	115	ENVCKKLDRI (SEQ ID NO: 712)	0.400
43	83	KLTQRIAQDV (SEQ ID NO: 713)	0.400
44	6	SLSSKNPASI (SEQ ID NO: 714)	0.400
45	69	KQTKLGCSKV (SEQ ID NO: 715)	0.400
46	38	VVPEPNLNEV (SEQ ID NO: 716)	0.400
47	180	KLYSLIGTTV (SEQ ID NO: 717)	0.400
48	92	VLRLSSTEPCL (SEQ ID NO: 718)	0.300

Table XVIII: HLA Peptide Scoring Results – 55P4H4 – B35, 10-mers			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
49	29	LSDFDYWDYV (SEQ ID NO: 719)	0.300
50	125	VCDSSVVPTF (SEQ ID NO: 720)	0.300

**Table XIX: Motif-bearing Subsequences of the 55P4H4 Protein**

**Protein Motifs**

5	N-glycosylation site	
	144-147	NCSW
10	Protein kinase C phosphorylation sites	
	1	8-10 SSK
	2	85-87 TQR
	3	149-151 SFR
15	Casein kinase II phosphorylation sites	
	1	14-17 SISE
	2	30-33 SDFD
	3	96-99 SSTE
	4	149-152 SFRD
20	N-myristoylation sites	
	1	5-10 GSLSSK
	2	102-107 GLRGCV

**Table XX: Frequently Occurring Motifs**

Name	av. % identity	Description	Potential Function
<u>zf-C2H2</u>	34%	Zinc finger, C2H2 type	Nucleic acid-binding protein functions as transcription factor, nuclear location probable
<u>cytochrome_b_N</u>	68%	Cytochrome b(N-terminal)/b6/petB	membrane bound oxidase, generate superoxide
<u>ig</u>	19%	Immunoglobulin domain	domains are one hundred amino acids long and include a conserved intradomain disulfide bond.
<u>WD40</u>	18%	WD domain, G-beta repeat	tandem repeats of about 40 residues, each containing a Trp-Asp motif. Function in signal transduction and protein interaction
<u>PDZ</u>	23%	PDZ domain	may function in targeting signaling molecules to sub-membranous sites
<u>LRR</u>	28%	Leucine Rich Repeat	short sequence motifs involved in

			protein-protein interactions
<u>pk</u> kinase	23%	Protein kinase domain	conserved catalytic core common to both serine/threonine and tyrosine protein kinases containing an ATP binding site and a catalytic site
<u>PH</u>	16%	PH domain	pleckstrin homology involved in intracellular signaling or as constituents of the cytoskeleton
<u>EGF</u>	34%	EGF-like domain	30-40 amino-acid long found in the extracellular domain of membrane-bound proteins or in secreted proteins
<u>rv</u> t	49%	Reverse transcriptase (RNA-dependent DNA polymerase)	
<u>ank</u>	25%	Ank repeat	Cytoplasmic protein, associates integral membrane proteins to the cytoskeleton
<u>oxidored_q1</u>	32%	NADH-Ubiquinone/plastoquinone (complex I), various chains	membrane associated. Involved in proton translocation across the membrane
<u>ef</u> hand	24%	EF hand	calcium-binding domain, consists of a 12 residue loop flanked on both sides by a 12 residue alpha-helical domain
<u>rv</u> p	79%	Retroviral aspartyl protease	Aspartyl or acid proteases, centered on a catalytic aspartyl residue
<u>Collagen</u>	42%	Collagen triple helix repeat (20 copies)	extracellular structural proteins involved in formation of connective tissue. The sequence consists of the G-X-Y and the polypeptide chains forms a triple helix.
<u>fn</u> 3	20%	Fibronectin type III domain	Located in the extracellular ligand-binding region of receptors and is about 200 amino acid residues long with two pairs of cysteines involved in disulfide bonds

7tm_1	19%	7 transmembrane receptor (rhodopsin family)	seven hydrophobic transmembrane regions, with the N-terminus located extracellularly while the C-terminus is cytoplasmic. Signal through G proteins
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**Table XXI: HOMOLOGY SEARCH RESULTS**  
**55P4H4 ORF ranges from base pairs 204-785.**

5 55P4H4 polynucleotides and polypeptides can be generated using the information provided in Table XXI. In particular, the polynucleotides and polypeptides identified in Table XXI can be used to identify parameters for the molecules of the present invention (e.g. the specific sequences and sizes of 55P4H4 polynucleotides and polypeptides not within the sequences identified below). For example, the 55P4H4 polynucleotides of the present invention can exclude any complete sequence or portion of one or more of the sequences identified below. Alternatively, the 55P4H4 polynucleotides of the present invention can be defined by a polynucleotide range based on the 5' and or 3' nucleotides of one or more of the sequences identified below. For example the 55P4H4 polynucleotides of the present invention include one or more polynucleotides having a sequence that begins at any 5' or 3' end of a first one of the sequences identified below and ends at any 5' or 3' end of a second one of the sequences identified below.

<u>dbEST database</u>		<u>base pair homology</u>	
gb AA528140.1 AA528140	nj15d07.s1 NCI_CGAP_Pr22 Homo sapien...	1047 0.0	2043-2590
gb AI375677.1 AI375677	ta58e04.x1 Soares_total_fetus_Nb2HF8...	948 0.0	2105-2590
20 gb AI670890.1 AI670890	wa06e03.x1 NCI_CGAP_Kid11 Homo sapie...	916 0.0	97-562
gb AI624970.1 AI624970	ts48f02.x1 NCI_CGAP_Ut1 Homo sapiens...	848 0.0	2155-2590
gb AI420574.1 AI420574	tf08a03.x1 NCI_CGAP_Pr28 Homo sapien...	821 0.0	2165-2590
gb AW237773.1 AW237773	xm81c03.x1 NCI_CGAP_Kid11 Homo sapie...	817 0.0	2139-2590
gb H15301.1 H15301	ym28a05.r1 Soares infant brain 1NIB Homo...	805 0.0	1612-2042
25 gb AI244780.1 AI244780	qj92f04.x1 NCI_CGAP_Kid3 Homo sapien...	660 0.0	2242-2590
emb Z43131.1 Z43131	HSC14B051 normalized infant brain cDNA ...	648 0.0	1579-1918
gb AI624952.1 AI624952	ts48d02.x1 NCI_CGAP_Ut1 Homo sapiens...	642 0.0	2267-2590
emb Z25007.1 Z25007	HSB83B122 STRATAGENE Human skeletal mus.583	e-164	1131-1465
gb AI758350.1 AI758350	ty68a03.x1 NCI_CGAP_Kid11 Homo sapie...	577 e-162	1255-1545
30 gb T30290.1 T30290	EST14265 Human Testis Homo sapiens cDNA ...	569 e-159	1524-1842
gb AA797319.1 AA797319	vw22g12.r1 Soares_mammary_gland_NbMM.484	e-134	358-777
gb H15695.1 H15695	ym28a05.s1 Soares infant brain 1NIB Homo...	478 e-132	2251-2572
gb AA472607.1 AA472607	vh04d09.r1 Soares_mammary_gland_NbMM..	420 e-115	
gb AI716417.1 AI716417	UI-R-Y0-abg-g-06-0-UI.s1 UI-R-Y0 Rat...	365 4e-98	
35 gb AA647389.1 AA647389	vq77c08.s1 Knowles Solter mouse 2 ce...	365 4e-98	
gb AA647405.1 AA647405	vq77e07.s1 Knowles Solter mouse 2 ce...	361 6e-97	
gb AA036156.1 AA036156	mi75f03.r1 Soares mouse p3NMF19.5 Mu...	351 6e-94	
emb Z39216.1 Z39216	HSC14B052 normalized infant brain cDNA ...	297 8e-78	
gb AI764467.1 AI764467	UI-R-Y0-abj-g-06-0-UI.s1 UI-R-Y0 Rat...	272 4e-70	
40 gb AI717465.1 AI717465	UI-R-Y0-acb-e-01-0-UI.s1 UI-R-Y0 Rat...	272 4e-70	
gb AI070152.1 AI070152	UI-R-Y0-lu-h-12-0-UI.s1 UI-R-Y0 Ratt...	196 2e-47	
gb AA095893.1 AA095893	l6491.seq.F Human fetal heart, Lambd...	180 1e-42	

gb|AI605099.1|AI605099 vh04d09.x1 Soares\_mammary\_gland\_NbMM... 168 5e-39  
 gb|AI454754.1|AI454754 UI-R-C2p-qj-h-04-0-UI.s1 UI-R-C2p Ra... 161 1e-36  
 gb|AA553003.1|AA553003 vk92d11.r1 Knowles Solter mouse 2 ce... 159 4e-36  
 dbj|AV044576.2|AV044576 AV044576 Mus musculus adult C57BL/6... 125 6e-26  
 5 dbj|AV208450.1|AV208450 AV208450 RIKEN full-length enriched... 115 6e-23  
 dbj|AV205532.1|AV205532 AV205532 RIKEN full-length enriched... 101 9e-19  
 gb|AI112228.1|AI112228 UI-R-Y0-mh-c-12-0-UI.s1 UI-R-Y0 Ratt... 96 6e-17  
 gb|AW431953.1|AW431953 73474 MARC 1BOV Bos taurus cDNA 5'. 74 2e-10  
 gb|AI171103.1|AI171103 EST217051 Normalized rat muscle, Ben... 44 0.18